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13. ABSTRACT (Maximum 200 words) Metastasis is a complex phenomenon involving both the activation and inactivation of a number of genes. Although a great deal is known about the types and numbers of genes that are activated or inactivated during metastasis, little is known about which genes are responsible for metastatic susceptibility. Identification and characterization of these genes would provide additional insights into this process, as well as potentially leading to novel therapeutic strategies. To identify inbred mouse strains harboring metastasis suppressors, previously we performed a survey to identify mouse strains that inhibited the ability of a transgene induced mammary tumor to form pulmonary metastases. Two inbred strains, DBA/2J and NZB/B1NJ were shown to significantly suppress the ability of the tumor to disseminate, indicative of the presence of metastasis suppressor genes in those genetic backgrounds. We have performed three independent genetic mapping experiments to determine the approximate genetic location of the metastasis suppressor genes. Our preliminary results suggest that there is at least one gene that is responsible the metastatic suppression, and possibly an unlinked second epistatically interacting gene. We are currently completing the preliminary mapping studies prior to initiating high resolution mapping strategies to further refine the genetic location(s) of the metastatic suppressor gene(s).				
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FOREWORD

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Introduction

The existence of metastatic suppressor genes was originally predicted based on somatic cell hybrid fusions between nonmetastatic and metastatic tumor cells. The resulting hybrids, while retaining their tumorigenic potential, were unable to metastasize [1-3]. A prostate cancer metastatic suppressor locus was localized on human chromosome 11 in the region 11p11.2, and was subsequently shown to be KAI1, a leukocyte surface glycoprotein [4]. Analysis of murine melanoma and human breast cancer cell lines revealed the specific down regulation of the gene NM23, a nucleoside 5'-phosphate kinase in metastatic tumors or cell lines versus nonmetastatic samples [5]. Introduction of E-cadherin cDNAs into tumor cell lines has demonstrated the suppression of metastatic capacity of both mouse and human carcinomas [6-8].

Additional evidence for the existence of genes that can suppress metastasis was generated from a series of transfection experiments into murine cells. It was determined that a variety of activated proto-oncogenes, including H-RAS, v-mos, v-raf, A-RAF, v-src, v-fes, v-fms, and p53, could induce primary tumors with metastatic dissemination when transfected NIH-3T3 cells were injected into mice. However, when the same oncogenes were transfected into cell lines derived from different strains of mice, metastatic potential, but not tumorigenicity, was lost [9, 10]. This suggests that certain alleles present in some of the inbred strains of mice, either alone or in combination, can function as a metastasis suppressor. At present, these loci have yet to be characterized.

Although the mouse has been used a model for a number of individual steps in the metastatic cascade, a mouse model for the entire process has not been developed. This is because naturally occurring mouse tumors in general do not metastasize, possibly due to the fact that the animal succumbs to the primary tumor before the metastatic process can be completed. A number of transgenic animals, however, have been found to metastasize, possibly due to the accelerated nature of the disease [11-22]. Since these animals develop metastatic disease in a heritable and highly penetrant manner, they offer the potential to utilize the power of mouse genetics to identify and characterize the modifier/suppressor loci known to be present in the mouse genome. One particularly interesting transgenic mouse is the mouse mammary tumor virus-polyoma middle T (MMTV-PyMT) transgenic animal, which develops mammary tumors and extensive pulmonary metastases [23]. The MMTV-PyMT transgenic mouse develops synchronously appearing multifocal tumors involving all of the mammary glands. Females develop palpable tumors within 8 weeks of birth, independent of pregnancy. Males also develop mammary tumors, although with a longer latency. In addition, more than 90% of the MMTV-PyMT animals develop hundreds of pulmonary metastases by 3 months of age. The high penetrance and extensive metastatic potential of this animal make it an excellent model to perform genetic screens for metastasis modifier/suppressor genes.

The purpose of this proposal is to utilize the MMTV-PyMT transgenic mouse as a model of human breast cancer metastasis in order to genetically localize, clone, and characterize genes that modulate or suppress the metastatic process. The differential ability of cell lines originating from different mouse strains to metastasize following the transfection of oncogenes suggests that there is at least one major metastatic suppressor gene present in some strains, although additional genes may be detected with a broad strain survey.

The specific aims of this project are as follows:

- 1) Utilize the highly metastatic MMTV-PyMT transgenic animal to perform a mouse strain survey to determine the mouse strains that contain dominant metastatic modifier/suppressor alleles by breeding the transgenic animal to a variety of mouse strains and scoring for latency, progression, extent and organ tropism of the mammary tumor metastases.
- 2) Select those mouse strains that demonstrate the largest effect on metastatic process and commence appropriate backcrosses to localize the gene or genes of interest as a preliminary step for isolation and characterization of the genes of interest, either by candidate gene or positional cloning strategies.
- 3) Initiate positional cloning projects to clone, identify and characterize the most promising candidate loci.

Body

A. Summary of Previous Work

The (FVB/N-TgN(MMTVPyMT)) transgenic animal was imported to the Fox Chase Cancer Center, and a colony established. The metastatic phenotype of our colony was assessed, was indistinguishable from the published phenotype, indicating that either the metastatic phenotype was independent of environmental factors, or that the same environmental factors existed in our animal facilities and the original facility. To develop a robust protocol for detecting metastatic suppresser genes in a mouse genetic screen, the affect of tumor duration, the period of time between diagnosis of the primary tumor and sacrifice, was determined. FVB/N-TgN(MMTVPyMT) virgin female animals were monitored until diagnosis of a primary mammary tumor, determined by daily visual inspection and palpation. The animals were subsequently aged to permit the potential development of metastases. The animals were then sacrificed and lungs were harvested from each of the animals and paraffin embedded for histological examination. Three nonadjacent sections from each lung, each separated by 100 microns, were hemotoxylin-eosin stained, and the pulmonary metastatic density was determined utilizing a Leica M420 low power microscope and the Q500MC Image Analysis System. The Leica Q500 Image Processing and Analysis System was utilized to exclude bronchial and alveolar space from the calculation, to minimize density variation that might arise from variable degrees of lung inflation at time of harvesting. Three randomly chosen fields were scored for each slide, for a total of 9 fields for each animal. Greater than 50% of the tissue area of each slide was estimated to have been scored. The number of metastases per unit area of lung was then plotted versus duration of tumor exposure. No significant trend was observed for increased tumor exposure. Since no significant difference was observed in the pulmonary metastatic density due to time, a time window of 30-40 days post-diagnosis was selected as the endpoint for the determination of pulmonary metastatic density.

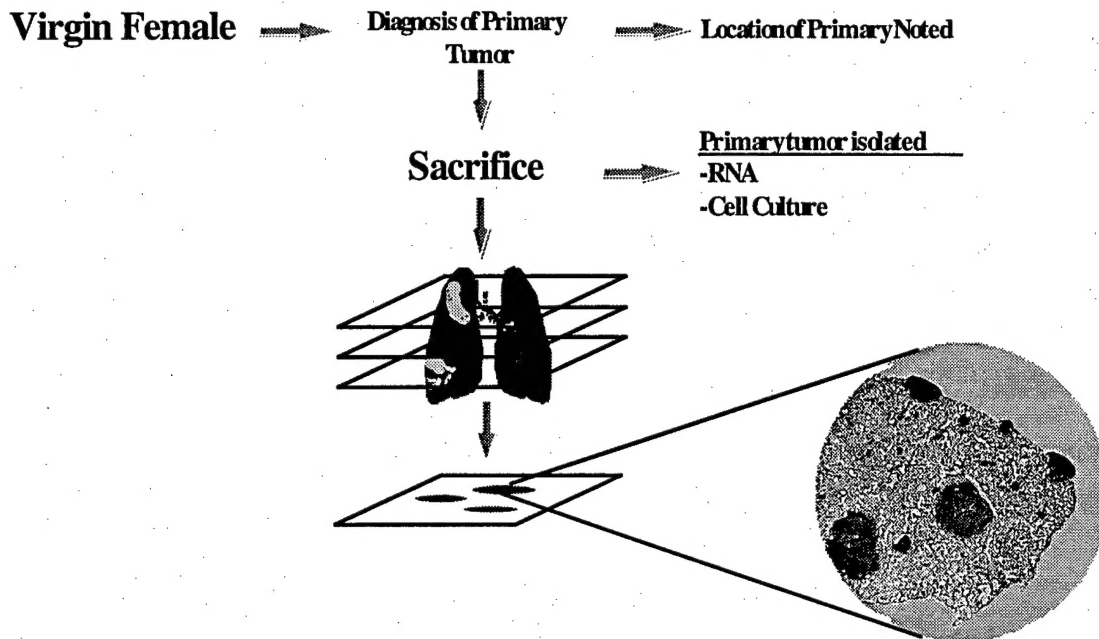


Figure 1: Protocol for Analysis of Pulmonary Metastasis. Virgin female transgenic animals are aged until induction of primary tumor, monitored to confirm presence of the tumor, and aged 30-40 days to permit metastatic progression. Primary tumor is harvested for Northern blot analysis and establishment of cell cultures. Lungs are harvested, sectioned, stained, and analyzed microscopically for number, size and density of pulmonary metastases.

To assess the effect of tumor burden on the metastatic phenotype, the correlation between pulmonary metastatic density and approximate tumor burden was determined. At diagnosis of the primary tumor, each animal was weighed, and then aged for 20-70 days. Animals were then sacrificed and total carcass weight was determined. A crude approximate of tumor burden was determined by subtracting weight at sacrifice by weight at diagnosis. Due to limited manpower and the large number of animals to be analyzed, it was not possible to dissect out the mammary tumors from all of the animals. Cachexia was not observed in any of the MMTVPyMT animals, therefore the change of weight should be a result predominantly, although not exclusively, of tumor tissue accumulation. In addition, most mouse strains accumulate body weight at approximately the same rate [24], so the percentage of the total change of weight that is tumor should be approximately the same in each strain. Complete dissection of the tumors from a subset of these animals demonstrated that on average greater than 75% of the change in weight was due to accumulation of tumor mass. Pulmonary metastatic density was determined from each animal, and plotted as a function of change in weight. In contrast with previous reports [25], a moderate correlation was observed between approximate tumor mass and pulmonary metastatic density ($r = 0.48$).

Identification of Inbred Strains Bearing Metastasis Suppressor Gene(s):

To identify mouse inbred strains bearing dominant metastasis suppresser alleles, the FVB/N-TgN(MMTVPyMT) animal was bred to 27 different inbred strains to generate F_1 animals. The inbred partners were selected from the various branches of the phylogenetic tree of the laboratory mouse [26], to increase the likelihood of observing a strain specific variation in metastatic potential. In addition to the common inbred strains, representative strains from the more evolutionary diverged species *M. castaneus*, *M. molossinus*, and *M. musculus* have also been included. Virgin female F_1 s were aged and monitored for the appearance of the primary tumor. Tumor bearing animals were maintained for 30-40 days after diagnosis, then sacrificed and autopsied. Representative animals from each mating were examined to determine whether metastatic organ tropism was altered. Primary tumor tissue was harvested to determine the transgene expression levels by northern blot analysis, and the lungs collected from each F_1 for histological examination as described previously. To control for the correlation between metastatic density and approximate tumor burden, the density of pulmonary metastases was normalized dividing the number of metastases per unit lung area by the approximate tumor burden, as defined by the change in weight.

As expected, a range of results was observed. A number of strains demonstrated no significant variation from the FVB/N parent strain in the number or size of the pulmonary metastases [27]. However, 14 different inbred strains had a statistically significant reduction in pulmonary metastatic density compared to FVB/N. Six strains demonstrate a 2-3 fold reduction, and the remaining 4 strains exhibit a 4-16 fold reduction. At present, none of the strains analyzed have demonstrated a significant increase in pulmonary metastases, suggesting the lack of a dominant metastatic enhancer allele in the inbred strains tested, relative to FVB/N. Northern and Western blot analysis of the PyMT transgene in the primary tumor indicate that there is not significant variation in expression between the FVB/N parent and the F_1 s, suggesting that the reduction in pulmonary metastatic density is not due to alterations in expression of the transgene. Ten strains accumulated statistically significantly less tumor burden, as measured by the average change of weight between diagnosis and sacrifice. The decrease in tumor tissue accumulation in these strains was the result of a combination of fewer tumors per animal as well as a decrease in tumor size. Sixteen strains accumulated tumor mass at an equal or greater amount than the FVB/N parental strain.

Significant suppression of pulmonary metastatic density was observed in the F_1 progeny of three inbred strains, DBA/2J, KK/HiH, and NZB/B1NJ (table 1). No differences were observed in the number of primary tumors or the average tumor burden. Therefore the decrease in the metastatic index in these strains is likely to be due to the presence of a metastatic suppressor gene(s) rather than a secondary effect of altered tumor initiation or growth (table 2). These three strains are distantly related, DBA/2J and NZB/B1NJ separated from common laboratory progenitors more than 50 years ago, and KK/HiJ derived from primarily Asian origin [28]. As a result of the distant relationship between these three strains, it was not possible to postulate whether the same metastatic suppresser gene(s) are present in all of the strains, or whether a different gene(s) are responsible for the phenotype observed.

Table 1: Affect of Maternal Genotype on Pulmonary Metastatic Density

Maternal Genotype	Metastatic Index	Standard Deviation	N	Metastatic Index Relative to FVB/N	p value versus FVB/N
FVB/N	3.31E-07	4.44E-07	79	1.00	1.00
NZB/B1NJ	2.68E-08	3.82E-08	31	0.08	6.4E-08
DBA/2J	4.30E-08	6.64E-08	21	0.13	1.7E-07
KK/HIJ	6.09E-08	6.71E-08	18	0.18	4.8E-07

Metastatic Index = number of metastases/lung area (cm^2)/tumor burden

P value was determined by comparing the FVB/N results to the average metastatic index for each strain with Student's T-test.

Table 2: Comparison of Average Change of Weight in F₁ Outcross Animals

Maternal Genotype	Average Change		Change Relative	
	in Weight (gms)	St Dev	to FVB/N	P value vs FVB/N
FVB/N	9.23	2.09	1.00	1.00
NZB/B1NJ	9.31	2.46	1.01	0.91
DBA/2J	9.53	3.97	1.03	0.80

P value was determined by comparing the FVB/N results to the average change of weight for each strain with Student's T-test.

Since the F₁ hybrid animals produced from inbred mouse strains DBA/2J and NZB/B1NJ demonstrated significant reduction in the density of pulmonary metastases but accumulate identical amounts of tumor tissue, with indistinguishable kinetics as the FVB/N animals, these strains were selected for quantitative trait mapping experiments. Two different strategies were pursued to map the genetic location of the metastatic suppressor loci. Preliminary low resolution mapping of the metastatic suppressor gene (or genes) in the DBA/2J strain is being carried out using the AKXD recombinant inbred mapping panel. Recombinant Inbred (RI) strains are mice that are derived from an outcross between two progenitor strains, for example AKR/J and DBA/2J, followed by an intercross. The F₂ animals are then randomly chosen and separated, then more than 20 generations of brother-sister matings performed. The result is a series of inbred strains whose genomes are mosaics of the progenitor strains, each line having a unique set of genes from each of the progenitor strains. Every RI strain therefore has a unique set of recombination sites distributed randomly throughout its genome. A set of RI strains can therefore be used essentially in the same manner as a mapping cross to determine linkage. RI strains have been very valuable for the analysis of complex traits, since a major advantage of RI analysis of complex traits is the ability to replicate the phenotype in unlimited number of animals for each RI genotype [29]. This permits the minimization of errors due to environmental or experimental variation by combining data from large numbers of animals to obtain a mean value for each RI genotype. This disadvantage of this strategy is that the small number of RI strains in each panel does not provide sufficient statistical rigor to definitively map loci unless the loci control a significant fraction of the trait's variance. Nonetheless, RI analysis can readily provide suggestive identification of genomic regions that contribute most strongly to the phenotype [30-32] that can subsequently be confirmed by backcross or intercross analysis. RI analysis is particularly useful because the segregation of the two parental genomes in each of

the common RI strains have already been determined. It is therefore possible to generate approximate genetic locations by determining the segregation of the phenotype in each RI line and comparing it to the pre-existing strain distribution pattern, without having to perform additional genotyping.

The second strategy currently in progress is the development of intraspecific backcrosses between FVB/N and both NZB/B1NJ and DBA/2J. Since an appropriate recombinant inbred panel does not exist for NZB/B1NJ, the generation of a backcross panel is required for the genetic mapping of the loci responsible for the suppression of the metastatic phenotype. In addition, due to the limited resolution of the recombinant inbred panel analysis, a backcross panel is being generated between FVB/N and DBA/2J to confirm the results of the recombinant inbred mapping experiment and to refine the genetic localization.

B. Progress during the Last Year

During the past 12 months we have initiated the three mapping experiments: AKXD RI panel, DBA/2J backcross, and the NZB/B1NJ backcross. The current status of each is reviewed below.

1) AKXD RI Mapping Experiment: The 21 strains that comprise the AKXD recombinant inbred panel were imported to Fox Chase Cancer Center. These animals were bred to the FVB-TgN(MMTV-PyMT) transgenic animal to generate transgene positive F_1 females for the mapping experiment. Progeny were successfully obtained from 18 of the 21 matings. Three of the AKXD RI strains failed to produce more than one offspring after multiple mating attempts. The F_1 progeny were aged as described above and tumor latency, tumor burden and pulmonary metastatic density determined. Unexpectedly, although the parental strains of the AKXD RI panel, DBA/2J and AKR/J, did not show any difference in accumulation of tumor tissue, 4 of the AKXD inbred strains demonstrated a statistically significant reduction in tumor growth. This result suggests that there is an epistatic interaction between loci present in the DBA/2J and AKR/J genetic backgrounds that influences the ability of the mammary tumors to grow. We have preliminary mapping data on the tumor growth loci and are currently assessing the statistical significance of our result.

The four RI strains in question accumulated only 50% of the tumor mass of the parental strains and the other members of the RI panel. These four RI strains also demonstrated a significant reduction in the number of pulmonary metastases as compared to the parental F_1 s or FVB/NJ. However, since we had previously demonstrated that total tumor burden at the time of sacrifice correlated, albeit weakly, with metastatic density we could not be certain that the decrease in the numbers of pulmonary metastasis was due directly to the decreased tumor burden, rather than a lower genetic susceptibility. Therefore to map the putative location of the metastasis susceptibility genes, these four RI strains were not included in the analysis. The loss of the four strains further reduced the power of the RI mapping experiment. However we anticipated that the RI experiment would still retain sufficient power to identify potential metastasis susceptibility genes that could be confirmed by analysis in the DBA/2J backcross.

The metastatic densities of the 14 remaining RI strains were analyzed using the MacIntosh program MapManager QTb21ppc to identify potential metastasis suppressor gene

map positions. Putative map positions were identified on 7 different chromosomes. As expected from the limited number of RI strains available from the AKXD panel, none of the 7 loci exceeded the statistically significant threshold used to positively identify quantitative trait loci. The significance of these loci are currently being assessed on the DBA/2J backcross to determine which loci are responsible for the metastatic suppression.

2) DBA/2J Backcross Mapping Experiment: FVB-TgN(MMTV-PyMT) males were bred to DBA/2J females and the transgene-positive F_1 males were subsequently backcrossed to transgene-negative FVB/NJ females to generate the N_2 backcross animals. A reciprocal backcross to DBA/2J was not performed due to the fear that there might be recessive alleles in the DBA/2J background that might alter the phenotype and thereby confuse the mapping results. Approximately 100 transgene-positive N_2 females were generated and scored for tumor latency, tumor growth and total tumor burden and pulmonary metastatic density. Unlike the AKXD RI mapping experiment, no statistically significant difference was observed between the N_2 animals and FVB-TgN(MMTV-PyMT) for either tumor latency or tumor growth and burden. The N_2 animals were then divided into high or low metastatic density classes. All animals whose metastatic density exceeded the median [DBA/2J x FVB-TgN(MMTV-PyMT)] F_1 value were considered to belong to the high metastatic class. Microsatellite loci from the putative metastatic suppressor regions suggested by the AKXD mapping experiment were used to haplotype the animals of both metastatic classes and the distribution of the haplotypes compared by Chi-Squared analysis.

To date we have completed the analysis of the 7 putative metastatic suppressor loci. Six of the loci did not display any segregation distortion in the N_2 animals, suggesting that the AKXD mapping result for those loci were false positives. The last locus on proximal chromosome 11 that we have examined, however, has demonstrated a correlation with metastatic density. Analysis of all of the animals in the backcross demonstrated a significant association of this locus with suppression of metastases ($p < 0.01$). The combined LOD score of the two experiments is approximately 3.1, approaching the statistically significant threshold of 3.3. At present we are adding additional markers in this region to confirm this result and potentially identify a region that is more highly associated with the metastatic suppression. In addition, to further assess the probability of a metastatic suppressor gene lying on this region of chromosome 11 we are generating additional N_2 animals to increase the power of our experiment. Our goal is to generate at least an additional 100 informative backcross animals. At present we have 81 new informative animals awaiting analysis. We anticipate completing the breeding in the next 2-3 months, and the analysis within the next 4-5 months.

3) NZB/B1NJ Backcross Experiment: FVB-TgN(MMTV-PyMT) males were bred to NZB/B1NJ females and the transgene-positive F_1 males were subsequently backcrossed to transgene-negative FVB/NJ females to generate the N_2 backcross animals. As with the DBA/2J backcross, a reciprocal backcross to NZB/B1NJ was not performed due to the fear that there might be recessive alleles in the NZB/B1NJ background that might alter the phenotype and thereby confuse the mapping results. A full genome scan was performed on 40 animals at either extreme of the metastasis density curve. 62 loci were typed on the 19 autosomes. Two loci have demonstrated a significant deviation from random segregation in the high versus low metastasis populations. A locus on proximal Chr. 9 from NZB/B1NJ appears to be significantly associated

metastatic suppression ($p < 0.001$). In addition, a locus on chromosome 13 also appears to be significantly associated with the metastatic phenotype. Analysis of the phenotype/genotype correlation demonstrated that the Chr. 9 locus haplotypes were opposite of what was expected. The low metastasis population had an over representation of the FVB/NJ rather than the NZB/B1NJ allele. This result suggests that the Chr. 9 allele acts interactively with the Chr. 13 allele, rather than as a single gene effect.

The 40 animals scored in the initial crosses do not provide enough statistical power to localize metastatic suppressor genes above the recommended statistical confidence threshold, although it provided valuable clues to potential regions of interest. We are therefore re-initiated the breeding of NZB/B1NJ N_2 animals in order to increase the statistical power of this cross. Our goal is to increase the number of informative animals to a total of 120-150. We anticipate that the breeding will be completed in the next 5-6 months, and the analysis of the modifier genes completed approximately 2 months subsequently.

Conclusions: Our data to date suggest the association of a locus on chromosome 11 in DBA/2J and Chr. 9 and 13 in the NZB/B1NJ inbred strain with metastatic susceptibility. We are currently performing confirmation experiments prior to initiating high resolution genetic mapping experiments as well as candidate gene analysis.

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Appendix

Research accomplishments

- Identified mouse inbred strains harboring metastatic suppressor alleles
- Identified mouse inbred strains harboring tumor latency modifier alleles
- Identified mouse inbred strains harboring tumor growth modifier alleles
- Identified polymorphic microsatellite loci between FVB/NJ, C58/J, I/LnJ, DBA/2J, MOLF/Ei, AKR/J, and NZB/B1NJ.
- Mapped two epistatically interacting loci that accelerate mammary tumor latency (manuscript submitted)
- Mapped two loci that suppress mammary tumor growth (manuscript in preparation)
- Tentatively identified two metastasis suppressor loci (work in progress)

Publications

1. Lifsted, T., Le Voyer, T., Williams, M., Muller, W., Klein-Szanto, A., Buetow, K.H. and Hunter, K.W. Identification of inbred mouse strains harboring genetic modifiers of mammary tumor age of onset and metastatic progression. *Intl. J. Cancer* 77:640-644, 1998.
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3. Le Voyer, T. and Hunter, K.W. Microsatellite DNA variants among the FVB/NJ, C58/J and I/LnJ mouse strains. *Mamm. Genome* 10:542-543, 1999.
4. Le Voyer, T., Lu, Z., Lifsted, T., Williams, M., Hunter, K. An epistatic interaction controls the latency of a transgene-induced mammary tumor. *Breast Cancer Res.* (submitted)

Meeting Abstracts

1. Lifsted, T., Williams, M., Hunter, K.W. A genetic screen for mammary tumor metastasis suppressor genes. 11th Intl. Mouse Genome Conference, St. Petersburg, FL, Oct. 12-16, 1997.
2. Lifsted, T., Williams, M., Muller, W., Hunter, K.W. A genetic screen for mammary tumor metastasis suppressor genes. The Mouse in Mammary Carcinogenesis Research Mtg., Jackson Lab, Bar Harbor, ME, Oct. 4-8, 1997.
3. Lifsted, T., Le Voyer, T., Williams, M., Muller, M., Klein-Szanto, A., Buetow, K.H. and Hunter K.W. Identification of inbred mouse strains harboring genetic modifiers of mammary

tumor age of onset and metastatic progression. Cancer Genetics & Tumor Suppressor Genes Meeting, Cold Spring Harbor, NY, August 19-23, 1998.

4. Lifsted, T., Le Voyer, T., Williams, M., Buetow, K.H. and. Hunter, K.W. Modifiers of Mammary Tumor Initiation. 12th International Mouse Genome Conference, Munich, Germany, September 30-October 4, 1998.
5. Le Voyer, T., Lu, Z., Lifsted, T., Williams, M. and Hunter, K. Initiation and progression of polyoma middle T-induced mammary tumors is controlled by multiple epistatically interacting loci. Mammary Carcinogenesis Research Mtg., Jackson Lab, Bar Harbor, ME, October 1999.
6. Le Voyer, T., Lu, Z., Lifsted, T., Williams, M. and Hunter, K. Initiation and progression of polyoma middle T-induced mammary tumors is controlled by multiple epistatically interacting loci. 13th Intl. Mouse Genome Conference, Philadelphia, PA, October 31-November 4, 1999.
7. Invited speaker, NCI Breast Cancer Think Tank, Alexandria, VA, July 29-30, 1999, "Mapping Modifiers of Mammary Tumor Initiation and Progression in the Mouse."

Personnel

Kent W. Hunter, Ph.D.

82. A GENETIC SCREEN FOR MAMMARY TUMOR METASTASIS SUPPRESSOR GENES.

Traci Lifstead, Max Williams, Kent Hunter. Fox Chase Cancer Center, Philadelphia, PA 19111

Metastasis is a complex phenomenon involving both the activation and inactivation of a number of genes. A great deal is known about the types and number of genes that are activated during the metastatic pathway, including proto-oncogenes, proteases, and growth factors. Relatively little is known, however, about the inactivation of genes that suppress the metastatic potential of tumor cells. The presence of metastatic modifier or suppressor genes has been demonstrated by a combination of somatic cell hybrid or transfection analysis.

To determine the efficacy of the mouse as a model system for the genetic analysis of metastasis suppressor genes, we have initiated a breeding experiment to determine whether dominant metastasis suppressor genes are present in different strains of inbred mice. The metastasis model utilized is a transgenic animal (FVB/N-TgN[MMTVPyMT]) that develops spontaneous multifocal mammary tumors and pulmonary metastases, the greater than 90% penetrance. Male transgenic animals were bred to more than 25 different inbred strains, and the effect of the maternal genome on pulmonary metastatic incidence determined. Statistically significant reduction in the number of pulmonary metastases has been observed for multiple inbred strains, suggesting that one or more metastasis suppressor genes were segregated into different inbred mouse strains during the genesis of the common laboratory strains.

One of the inbred strains with the greatest effect on metastatic progression is DBA/2J. Pulmonary metastatic density in (FVB/N-TgN[MMTVPyMT] x DBA/2J)F1s is reduced greater than 12-fold compared to the FVB/N-TgN[MMTVPyMT] parent ($p < 10^{-7}$). Since (AKR/J x FVB/N-TgN[MMTVPyMT])F1s are phenotypically indistinguishable from the transgenic parent, and therefore do not carry metastatic suppressor alleles, preliminary mapping of the DBA/2J metastatic suppressor genes is being performed with the AKXD RI lines. A QTL backcross panel is also currently being developed.

Hunter, Kent
The Mouse in Mammary Carcinogenesis Research
The Jackson Laboratory
Bar Harbor, ME
Oct. 4-8, 1997

A GENETIC SCREEN FOR MAMMARY TUMOR METASTASIS SUPPRESSOR GENES.

Traci Lifestad¹, Max Williams¹, William Muller, and Kent Hunter¹. ¹Fox Chase Cancer Center, Philadelphia, PA 19111, and ²McMaster University, Toronto, Ontario, Canada.

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Cancer Genetics & Tumor Suppressor Genes
Cold Spring Harbor, NY
August 19-23, 1998

Hunter

Oral

IDENTIFICATION OF INBRED MOUSE STRAINS HARBORING GENETIC MODIFIERS OF MAMMARY TUMOR AGE OF ONSET AND METASTATIC PROGRESSION

Traci Lifsted, Thomas LE Voyer, Max Williams, William Muller*, Andres Klein-Szanto, Kenneth H. Buetow, and Kent W. Hunter Division of Population Science, Fox Chase Cancer Center, Philadelphia, PA, USA and *McMaster University, Hamilton, Ontario, Canada.

Metastatic progression is a major cause of mortality in cancer patients. Dissemination of the primary tumor throughout the body often precludes surgical removal, and these metastatic lesions many times are refractory to chemotherapeutic strategies. As a result, a significant fraction of cancer patients succumb to metastatic burden, or to complications of antimetastatic therapy. Comprehensive understanding of metastatic progression would be of enormous benefit for the prevention and treatment of disseminated tumors. However, due to the complexity of the process, involving many regulatory events (both positive and negative), the process of metastasis is poorly understood.

Since quantitative trait or complex trait genetic mapping has been successfully used to identify genes that modify mouse models of complex human traits including ethanol addiction, diabetes and susceptibility to tumorigenesis, we hypothesized that this strategy would be useful for the identification of genes that modify the ability of a tumor to metastasize. To identify mouse strains that might harbor dominant genetic modifiers of mammary tumor metastasis, we performed a strain survey utilizing a transgenic mouse mammary tumor model that exhibits a high incidence of pulmonary metastases. The transgenic animal was bred to 27 different inbred strains of mice, and scored for the metastatic organ tropism and metastatic density. Thirteen strains were identified that had a statistically significant reduction in the numbers of pulmonary metastases. In addition, 10 strains were identified that altered the kinetics of induction of the primary mammary tumor. These strains will provide useful model systems for the analysis of genetic interactions in the initiation and progression of mammary adenocarcinomas.

12th International Mouse Genome Conference

Garmisch, Germany

September 29-October 3, 1998

Modifiers of Mammary Tumor Initiation. Traci Lifsted, Thomas Le Voyer, Max Williams, Kenneth H. Buetow, and Kent W. Hunter Division of Population Science, Fox Chase Cancer Center, Philadelphia, PA, USA.

The inherited component of breast cancer was originally observed more than 100 years ago and has been confirmed by the identification of two major susceptibility genes, *BRCA1* and *BRCA2*. Women with germline mutations in these loci have a greatly enhanced risk of breast cancer. The likelihood of women carrying mutations in *BRCA1* approaches approximately 80% by the age of 70 and carriers are also at increased risk for ovarian cancer. Approximately 70% of breast cancer families can be accounted for by these high susceptibility genes and between 10 -15% of breast cancer in the general population.

Although the discovery of these high susceptibility genes is important for understanding the genetic basis of breast cancer many important questions remain to be answered. The clinical expression of *BRCA1* has been shown to vary both between families and within families. Some women may develop breast cancer in early in life, while family members bearing the same mutation remain unaffected until their seventies. The variable penetrance and age-at-onset observed between individuals bearing the same mutations suggests that there must be additional factors that influence the development of the disease. At present, however, little is known about breast cancer modifier genes and how they interact with the major susceptibility genes. Due to the genetic heterogeneity of the human population and interactions with uncontrolled environmental influences, identification of modifiers in human populations can be a difficult task.

To identify the genetic factors that influence the age of onset of mammary tumor initiation our laboratory studies the FVB/N-TgN(MMTVPyMT) transgenic mouse. This animal bears the polyoma middle T antigen whose expression results in the development of palpable synchronous multifocal tumors at approximately 60 days of age. The mammary tumors replicate many of the biochemical and histological events of some breast cancers. To identify inbred strains bearing genes that modify the dominantly expressed tumor phenotype, we have performed a mouse strain survey by breeding this transgenic animal to 27 different inbred strains from different branches of the mouse phylogenetic tree and determining the average age of tumor induction. The primary tumors in the F₁ progeny of two inbred strains, C58/J and I/LnJ, were detected approximately two and a half weeks earlier than the FVB/N transgenic parent strain. In addition, the F₁ progeny of 6 strains, SWR/J, AKR/J, BUB/BnJ, ST/J, KK/Hij and MOLF/Ei, developed palpable tumors approximately 1 to 5 weeks later than the FVB/N-TgN(MMTVPyMT). These data strongly suggest the presence of genetic modifiers of mammary tumor initiation in this model system. We are currently developing backcrosses to map the loci responsible for the alteration in mammary tumor latencies.

Hunter, Kent
Mammary Cancer Meeting
Jackson Lab, Bar Harbor, ME
October, 1999

INITIATION AND PROGRESSION OF POLYOMA MIDDLE T-INDUCED MAMMARY TUMORS IS CONTROLLED BY MULTIPLE EPISTATICALLY INTERACTING LOCI. Le Voyer, T., Lu, Z., Lifsted, T., Williams, M. and Hunter, K. Fox Chase Cancer Center, Philadelphia, PA, USA.

Previous studies from our laboratory demonstrated that introduction of the I/LnJ inbred genetic background into the FVB/NJ-PyMT animal significantly accelerates the appearance of the primary tumor (35 vs. 57 days postnatal, $p < 10^{-7}$). A backcross mapping panel was established and loci responsible for the tumor acceleration were detected on chrs. 15 and 9. Examination of the genotype/phenotype correlation revealed that the FVB/NJ but not the I/LnJ allele of the chr. 15 locus was associated with tumor acceleration, but was conditional on the presence I/LnJ allele on chr. 9. These loci, designated *Apmt1* and *Apmt2* map to syntenic regions associated with LOH in human breast cancer. These results suggest that in addition to loss of function, allelic variants of genes in these regions may contribute to age of onset in human breast cancer.

INITIATION AND PROGRESSION OF POLYOMA MIDDLE T-INDUCED MAMMARY TUMORS IS CONTROLLED BY MULTIPLE EPISTATICALLY INTERACTING LOCI. Le Voyer, T., Lu, Z., Lifsted, T., Williams, M. and Hunter, K.
Fox Chase Cancer Center, Philadelphia, PA, USA.

Previous studies from our laboratory demonstrated that the latency, tumor growth and metastatic progression of polyoma middle T-induced mammary tumor in an FVB/NJ inbred mouse background could be significantly altered by the introduction of different genetic backgrounds. In this study we extend these findings by mapping a number of interacting quantitative trait loci responsible for the changes in phenotype.

Introduction of the I/LnJ inbred genetic background into the FVB/NJ-PyMT animal significantly accelerates the appearance of the primary tumor (35 vs. 57 days postnatal, $p < 10^{-7}$). A backcross mapping panel was established and loci responsible for the tumor acceleration were detected on chrs. 15 and 9. Examination of the genotype/phenotype correlation revealed that the FVB/NJ but not the I/LnJ allele of the chr. 15 locus was associated with tumor acceleration, but was conditional on the presence of the I/LnJ allele on chr. 9. These loci, designated *Apm1* and *Apm2* map to syntenic regions associated with LOH in human breast cancer. These results suggest that in addition to loss of function, allelic variants of genes in these regions may contribute to age of onset in human breast cancer.

In addition to the acceleration of the tumor latency, introduction of the I/LnJ background into the FVB/NJ-PyMT mouse significantly suppresses the growth of the tumors. F_1 animals between FVB/NJ-PyMT and I/LnJ accumulate only ~half the tumor tissue of the FVB/NJ-PyMT parent. Analysis of microvessel density of the tumors did not reveal a significant difference between the two genotypes. In addition, no significant differences in apoptosis have been detected in preliminary studies. A genome wide screen in the backcross revealed three loci significantly associated with tumor growth suppression. Two loci were present on proximal or central chr. 4 and one locus on proximal chr. 7. Examination of the genotype/phenotype correlation revealed that like *Apm1* the FVB/NJ not the I/LnJ allele on chr. 7 was significantly associated with tumor suppression, and probably requires the FVB/NJ allele at one or both of the loci on chr. 4.

IDENTIFICATION OF INBRED MOUSE STRAINS HARBORING GENETIC MODIFIERS OF MAMMARY TUMOR AGE OF ONSET AND METASTATIC PROGRESSION

Traci LIFSTED¹, Thomas LE VOYER¹, Max WILLIAMS¹, William MULLER², Andres KLEIN-SZANTO¹, Kenneth H. BUETOW¹ and Kent W. HUNTER^{1*}

¹Division of Population Science, Fox Chase Cancer Center, Philadelphia, PA, USA

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Metastasis is one of the most important and complex processes in human neoplastic disease. A large number of both positive and negative events must occur to permit a tumor cell to colonize a distant site successfully. To identify mouse strains that harbor dominant genetic modifiers of this process, a strain survey was initiated utilizing a transgenic mouse mammary tumor model that exhibits a high incidence of pulmonary metastases. The transgenic animal was bred to 27 different inbred strains of mice and scored for the metastatic organ tropism and metastatic density. Thirteen strains were identified that had a statistically significant reduction in the numbers of pulmonary metastases. In addition, 10 strains were identified that altered the kinetics of induction of the primary mammary tumor. These strains will likely provide useful model systems for the analysis of genetic interactions in the initiation and progression of mammary adenocarcinomas. *Int. J. Cancer* 77:640–644, 1998.

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The mouse has been increasingly utilized as a model for the analysis of complex genetic human phenotypes (Frankel, 1995). The mouse models of human complex traits that have been successfully developed to date include ethanol addiction (Belknap *et al.*, 1993), diabetes (de Gouyon *et al.*, 1993), susceptibility to tumorigenesis (Ghosh *et al.*, 1993), kidney disease (Iakoubova *et al.*, 1995), neural tube defects (Neumann *et al.*, 1994) and obesity (Warden *et al.*, 1995). The availability of high-resolution mouse genetics and the large number of well-characterized inbred strains make the mouse a powerful system for studying multigenic or quantitative trait human disease phenotypes (Frankel, 1995).

A particularly important complex disease phenotype is that of tumor dissemination, or metastasis. Metastasis is one of the most important aspects of neoplastic disease and one of the most poorly understood (for review, see Liotta and Stetler-Stevenson, 1993). Although significant progress has been made in the elucidation of the metastatic process, a comprehensive understanding has been hampered by the complexity of the process and the intricacies of the tumor-host interaction (Liotta and Stetler-Stevenson, 1993). It has become apparent that development of metastatic disease is a multistep process that requires both positive and negative regulatory events to complete the metastatic cascade. Positive events include the activation of oncogenes and growth factors and induction of proteases, cellular adhesion proteins, motility factors and angiogenesis. Negative, or loss of function, events include inactivation of tumor suppressor genes and growth inhibitor processes, and the loss or inactivation of metastatic suppressors (Liotta and Stetler-Stevenson, 1993; Welch *et al.*, 1994).

Evidence for the genetic control or modulation of metastasis was generated from a number of studies, utilizing somatic cell hybrid fusions between non-metastatic and metastatic tumor cells (Miele *et al.*, 1996; Ramshaw *et al.*, 1983; Welch *et al.*, 1994). The resulting hybrids, while retaining their tumorigenic potential, were unable to metastasize. Subsequently, a prostate cancer metastatic suppressor locus was localized on human chromosome 11 in the region 11p11.2 and was eventually shown to be KAI1, a leukocyte surface glycoprotein (Dong *et al.*, 1995). Analysis of murine melanoma and human breast cancer cell lines have also revealed the specific down-regulation of the gene NM23, a nucleoside

5'-phosphate (NDP) kinase in metastatic tumors or cell lines vs. non-metastatic samples (Steege *et al.*, 1991).

Additional evidence for the genetic modulation of metastasis was obtained by a series of transfection experiments into murine cells (Tuck *et al.*, 1990). It was determined that a variety of activated proto-oncogenes, including H-RAS, v-mos, v-raf, A-RAF, v-src, v-fes, v-fms and p53, can induce tumorigenicity and metastatic potential when transfected into NIH-3T3 cells. However, when the same oncogenes were transfected into cell lines derived from different strains of mice, metastatic potential, but not tumorigenicity, was lost (Tuck *et al.*, 1990). This suggests that certain alleles present in some of the inbred strains of mice, either alone or in combination, can function as a metastasis suppressor. At present, these loci have yet to be characterized.

Although the mouse has been used as a model for a number of individual steps in the metastatic cascade, a mouse model for the entire process has not been developed. This is possibly a result of the fact that spontaneous metastatic mouse tumors have long latencies (Liebelt *et al.*, 1968), and the animals may succumb to the primary tumor or require sacrifice before the metastatic process can be completed. In addition, the poor penetrance of the metastatic phenotype of spontaneous mouse tumors (Liebelt *et al.*, 1968) would significantly complicate the analysis. A number of transgenic animals, however, have been found to metastasize extensively, possibly due to the accelerated nature of the disease (Anand *et al.*, 1994; Guy *et al.*, 1992; Nielson *et al.*, 1995; Wilkie *et al.*, 1994; Yang *et al.*, 1995). Since these animals develop metastatic disease in a heritable and highly penetrant manner, they offer the potential to utilize the power of mouse genetics to identify and characterize the modifier/suppressor loci known to be present in the mouse genome. One particularly interesting transgenic mouse is the MMTV-polyoma middle T (MMTV-PyMT) transgenic animal, which develops mammary tumors and extensive pulmonary metastases (Guy *et al.*, 1992). The MMTV-PyMT transgenic mouse develops synchronously appearing multifocal tumors involving all the mammary glands. Females develop palpable tumors within 60 days of birth, independent of pregnancy. Males also develop mammary tumors, although with a longer latency. In addition, more than 90% of the animals develop pulmonary metastases by 100 days (Guy *et al.*, 1992). The high penetrance and extensive metastatic potential of this animal make it an excellent model to perform genetic screens for metastasis modifier/suppressor genes.

To develop a model system for identification and characterization of dominant metastasis modifier/suppressor genes, our labora-

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tory has performed a genetic survey of more than 25 inbred strains. Virgin female transgene-positive F_1 progeny from (MMTV-PyMT \times inbred) outcrosses were generated and aged for the induction of the primary tumor and subsequent metastasis. The F_1 progeny were subsequently scored for metastatic target organ and density of pulmonary metastases. Thirteen inbred strains were identified that demonstrated a statistically significant reduction in the density of pulmonary metastases. Unexpectedly, 10 inbred strains were also identified that demonstrated significantly different latencies in the development of the primary mammary tumors. Utilization of these inbred strains and the MMTV-PyMT transgenic mouse in quantitative trait and standard genetic mapping experiments will likely provide novel genes for analysis of the complex process of mammary tumor formation and malignant progression.

MATERIAL AND METHODS

Mice

The FVB/N-TgN(MMTVPyMT) transgenic mouse utilized was the 634 line developed in the laboratory of W. Muller. I/LnJ, C58/J, ST/J, KK/HIJ, BUB/BnJ, NOD/LJ, MOLF/Ei, SWR/J, AKR/J, CBA/CaJ, NZW/LacJ, DBA/1J, DBA/2J, 129/J, P/J, SEA/GnJ, A/J, LP/J, CE/J, RF/J, C57BL/10J, NZB/B1NJ, FVB/NJ and C57BR/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). CAST/Ei was a kind gift from Dr. M. Brilliant, Fox Chase Cancer Center (Philadelphia, PA). C57BL/6JNcr, BALB/cAnNcr and C3H/HeNcr mice were purchased from the Laboratory Animal Facility of the Fox Chase Cancer Center. Tail biopsies were taken from weanlings to screen for germline transmission of the transgene. The MMTVPyMT was detected by PCR amplification with the following primers: 5'-AAC GGC GGA GCG AGG AAC TG-3'; 5'-ATC GGG CTC AGC AAC ACA AG-3'.

Strain survey

FVB/N-TgN(MMTVPyMT) males were bred to females from each of the inbred strains. Female transgenic F_1 s were screened by palpation 3 times a week for the presence of the primary mammary tumor. The location of the tumor and the weight of the animal were recorded. The animals were examined for an additional week to confirm diagnosis and then aged for 40 days post-diagnosis to permit development of metastases. After 40 days, the animals were sacrificed by carbon dioxide inhalation; total carcass weight was determined, autopsies performed, and the lungs harvested for histological examination.

Histological analysis

Tissues were fixed in 10% paraformaldehyde, embedded in paraffin, serial sectioned and hematoxylin-eosin stained. For the determination of pulmonary metastatic density, 3 coronal non-adjacent sections of both lungs, each separated by 100 μ m, were prepared from each animal. The slides were examined with a Leica M420 Macroviewer with an Apozoom lens under 10 \times magnification with the objective 10 cm above the stage. Three fields were scored for each slide, for a total of 9 fields per animal. Pulmonary metastatic density was determined utilizing a Leica Q500MC Image Analysis System. All slides were read blind, and analysis was performed by a single operator to minimize operator bias. The macro used is available upon request.

Northern blot analysis

RNA was isolated from primary tumor tissue using RNeasy Maxi Kit (Qiagen, Santa Clara, CA), following the manufacturer's protocol; 15 μ g of total cellular RNA per sample were fractionated on 1% formaldehyde gels, stained with EtBr, and transferred to Hybond-N⁺ (Amersham, Arlington Heights, IL) by capillary transfer. Probes were labeled with the Prime-It kit (Stratagene, La Jolla, CA) and hybridized in Church's solution, as described by Church and Gilbert (1984).

RESULTS

Preliminary characterization of metastatic variables

To determine the variables that influence the metastatic phenotype, a preliminary characterization of the effect of length of tumor exposure, and of approximate tumor burden, on the density of pulmonary metastases was performed. Virgin FVB/N-TgN(MMTVPyMT) females were generated and monitored for the appearance of the primary tumor. At diagnosis of the primary tumor, each animal was weighed, and then aged for 20–70 days. Animals were then sacrificed, and total carcass weight was determined. A crude approximate of tumor burden was determined by subtracting weight at sacrifice from weight at diagnosis. No cachexia was observed in the MMTVPyMT animals; therefore, the change of weight should be a result predominantly, although not exclusively, of tumor tissue accumulation. Pulmonary metastatic density was determined from each animal and plotted as a function of either tumor period (defined as number of days between diagnosis and sacrifice) or tumor burden. In agreement with previous studies (Liebelt *et al.*, 1968), no significant correlation with tumor period and pulmonary metastatic density was observed (data not shown). In contrast to previous reports (Liebelt *et al.*, 1968), a weak correlation was observed ($r = 0.49$), however, between approximate tumor mass and pulmonary metastatic density (Fig. 1).

Strain survey

To identify inbred mouse strains that harbor dominant genetic modifiers of mammary gland carcinogenesis and progression, the MMTVPyMT animal was bred to 27 different inbred strains of mice. The inbred strains from different branches of the mouse phylogenetic tree and ferally derived strains were selected to maximize the probability of detecting strain-specific variations. Transgene-positive female F_1 s from each cross were monitored for the appearance of the primary tumor and then aged approximately 40 days to permit metastatic progression. The approximate tumor burden was calculated as described above. Three phenotypes were scored for each animal: tumor latency, metastatic target organ and density of metastases. No strain-specific variation in metastatic organ was observed. Significant variation was observed in the latency of the primary tumors (Table I) for a number of strains. Two strains, I/LnJ and C58/J, demonstrated statistically significant acceleration (20.6 and 17.8 days earlier, respectively; $p < 10^{-15}$ and 10^{-10} , respectively) of the primary tumor compared with the FVB/N-TgN(MMTVPyMT) animal. Seven additional strains demonstrated statistically significant delays of ≥ 7 days, compared with FVB/N, in the appearance of the primary tumor (C57BL/6JNcr, NOD/LJ, SWR/J, AKR/J, BUB/BnJ, ST/J, KK/HIJ and MOLF/Ei; $p < 0.01 - 10^{-6}$).

Histological examination of the lungs was performed utilizing a Leica Q500MC Image Analysis System to quantitate the number of metastases in each sample. The results were normalized to the amount of lung tissue scored to reduce errors due to variations in sample sizes on each slide. Since a weak correlation between the number of metastases and the amount of primary tumor tissue was observed (Fig. 1), the density of metastases (defined as the number of metastases per unit area of lung) was normalized to the approximate tumor burden. The resulting data, termed the metastatic index, was averaged for each inbred strain combination and compared with the FVB-TgN(MMTVPyMT) by Student's *t*-test to determine statistical significance (Table II). Thirteen inbred strains demonstrated statistically significant reduction of the density of pulmonary metastases compared with the FVB-TgN(MMTVPyMT) parent strain, with reductions ranging from 2- to 25-fold. Only one strain, AKR/J, demonstrated a consistent, although not statistically significant, increase in the density of pulmonary metastases, of approximately 3-fold.

Analysis of transgene expression

To determine whether the change in latency of the primary tumor or the change in pulmonary metastatic density could be correlated

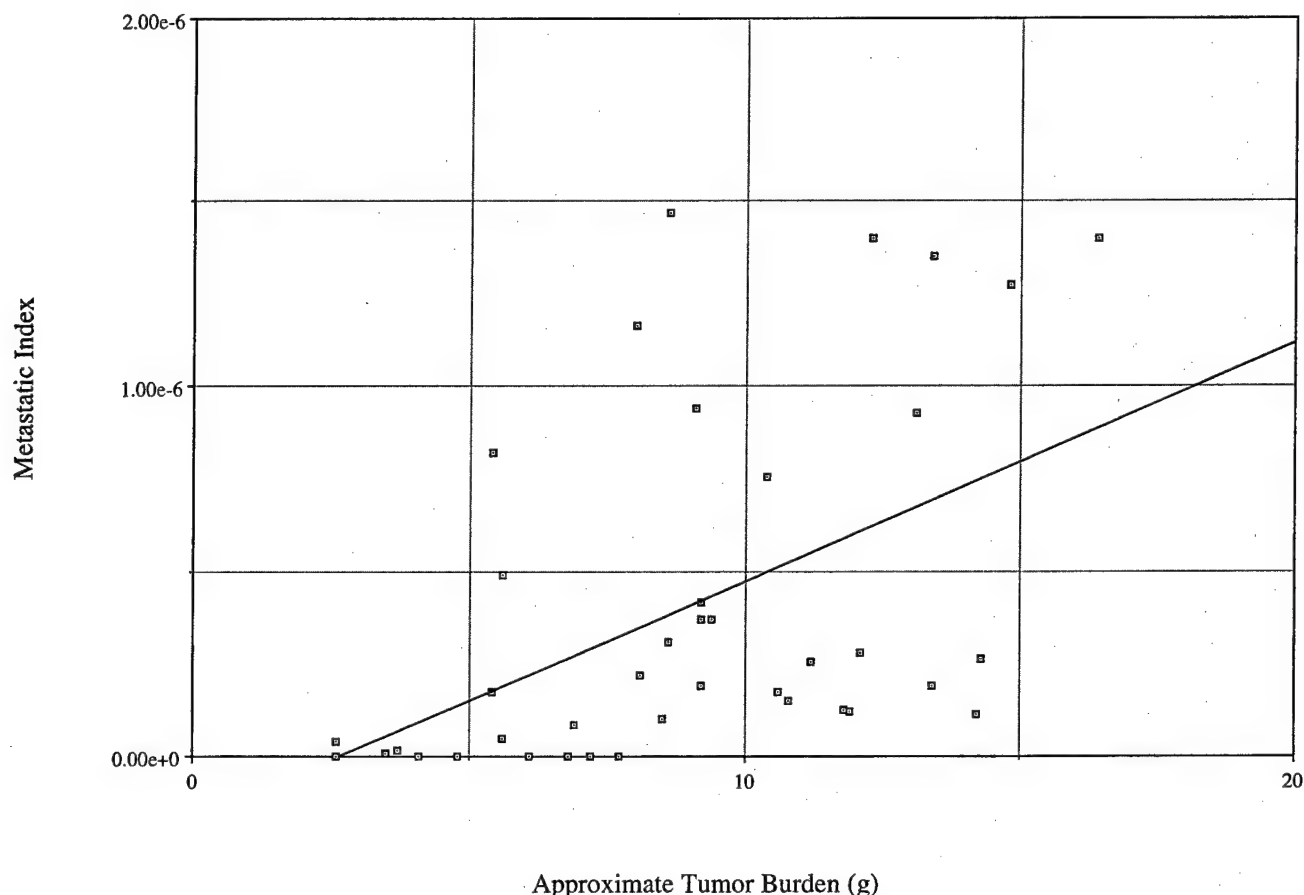


FIGURE 1 – Effect of approximate tumor mass, as measured by change in weight, on metastatic index. FVB/N transgenic females were aged for various periods after diagnosis of the primary tumor and sacrificed; approximate tumor burden and pulmonary metastatic densities were then determined. The data were entered into the program Cricket Graph v. 1.3.2 and analyzed as a scatterplot, and a best fit line was calculated.

with level of transgene expression, Northern blots were performed. Total cellular RNA from representative primary tumors from animals were fractionated on formaldehyde gels and hybridized with PyMT and β -actin probes. No correlation of increased transgene expression with decreased latency or reduction of metastatic index was observed (Fig. 2). In fact, a slight decrease in transgene expression was observed in the I/LnJ and C58/J F_1 tumors analyzed, suggesting that the decreased latency was due to additional modifier genes, rather than a direct effect of transgene expression.

DISCUSSION

Our study was designed to identify dominant genetic modifier alleles that modulate the ability of tumors to metastasize. Several variables that might influence the metastatic phenotype, including length of tumor exposure and approximate tumor mass, were analyzed in FVB/N-TgN(MMTVPyMT) animals to determine the appropriate endpoint and analytical methods. As expected, no significant correlation was observed between the length of time exposed to the tumor (defined as number of days between diagnosis of primary tumor and sacrifice) and the density of pulmonary metastases. This suggests that although the expression of the MMTVPyMT transgene is likely sufficient to induce the primary tumor, transgene expression does not provide all the additional signals or events required for metastatic progression. Since all these animals are presumably expressing the transgene with identical temporal kinetics, random secondary events must be

occurring to explain the wide variance observed in metastatic ability. Since tumor period did not significantly influence the density of metastases observed, 40 days after diagnosis of the primary tumor was selected for the experimental endpoint.

The second variable that might influence the measured metastatic ability was the approximate total tumor mass. A previous study had not demonstrated a correlation with the number of metastases and the amount of spontaneously arising primary tumors (Liebelt *et al.*, 1968). We performed preliminary experiments to determine whether there might be a correlation in this transgenic model. In contrast to the previous study, a weak correlation was observed. A possible explanation of the correlation would be that the greater the potential target tissue, the greater the probability of successfully completing the multiple steps required for metastatic progression. Therefore, all the determination of metastatic density index was normalized to approximate tumor mass to account for this weak correlation. The tumor mass utilized in this study is only an approximation. Due to the number of animals involved in our study, it was not feasible to dissect all the tumor tissue from each of the more than 400 animals studied. Since cachexia was not observed in the FVB/N parent or any of the outcross progeny, we believe that the change in weight of the animals between primary diagnosis and sacrifice is predominantly due to tumor tissue, with 2 notable exceptions. These are the F_1 progeny of the cross to I/LnJ and C58/J. I/LnJ and C58/J develop primary tumors prior to achieving adult size, and therefore the tumor burden as measured reflects both tumor tissue and normal

TABLE I - EFFECT OF MATERNAL GENOTYPE ON PRIMARY TUMOR LATENCY

Maternal genotype	Average latency (days)	SD	Median age (days)	Change in latency (days)	p value vs. FVB
I/LnJ	37.32	6.84	36	-20.60	2.17E-16
C58/J	40.11	6.82	39	-17.81	3.66E-11
LP/J	51.25	17.29	50	-6.67	0.45
129/J	53.58	12.43	51.5	-4.34	0.20
A/J	53.83	12.40	52	-4.09	0.39
BALB/cAnNlcr	56.44	9.96	58	-1.49	0.43
C3H/HeNlcr	57.80	13.10	58	-0.12	0.80
FVB/N	57.92	9.93	57	0.00	1.00
DBA/2J	59.44	12.03	59	1.51	0.84
NZB/B1NJ	59.64	7.86	61	1.72	0.68
C57BR/cdJ	59.88	11.42	62.5	1.95	0.73
C57BL/10J	60.31	11.54	59	2.38	0.66
CE/J	60.45	7.05	61	2.53	0.51
RF/J	61.20	8.23	64	3.28	0.56
SEA/GnJ	61.25	9.09	61.5	3.33	0.29
P/J	61.47	7.28	62	3.55	0.22
DBA/1J	64.00	11.59	62	6.08	0.19
NZW/LacJ	64.00	11.51	68	6.08	0.18
C57BL/6JNlcr	64.44	10.54	66	6.51	0.01
CAST/Ei	65.17	9.56	66	7.24	0.17
NOD/LtJ	66.65	9.53	69	8.73	3.95E-03
CBA/CaJ	66.67	13.08	68.5	8.74	0.07
SWR/J	66.74	14.46	67	8.82	9.71E-03
AKR/J	68.54	11.74	67	10.62	0.01
BUB/BnJ	69.64	7.64	68.5	11.72	1.64E-04
ST/J	74.00	8.79	77	16.08	2.69E-06
KK/HlJ	76.00	12.25	76	18.08	7.28E-05
MOLF/Ei	80.63	16.78	83.5	22.70	7.38E-03

TABLE II - EFFECT OF MATERNAL GENOTYPE ON PULMONARY METASTATIC DENSITY

Maternal genotype	Metastatic index	SD	No.	Metastatic index ¹ relative to FVB/N	p value vs. FVB/N
FVB/N	3.58E-08	4.60E-08	45	1.00	1.00E+00
RF/J	8.63E-10	1.93E-09	5	0.02	ND
C58/J	1.38E-09	2.19E-09	17	0.04	9.43E-06
C57BR/cdJ	2.35E-09	4.16E-09	16	0.07	1.52E-04
NZB/B1NJ	2.68E-09	3.82E-09	23	0.07	1.82E-05
I/LnJ	2.94E-09	6.15E-09	18	0.08	2.37E-05
DBA/2J	4.30E-09	6.64E-09	14	0.12	5.02E-05
KK/HlJ	6.09E-09	6.71E-09	13	0.17	1.19E-04
MOLF/Ei	7.08E-09	8.61E-09	5	0.20	ND
SEA/GnJ	8.39E-09	1.12E-08	23	0.23	2.82E-04
NZW/LacJ	1.24E-08	2.01E-08	10	0.35	0.02
CE/J	1.27E-08	1.42E-08	11	0.35	6.17E-03
ST/J	1.37E-08	2.01E-08	11	0.38	3.47E-04
C57BL/6JNlcr	1.47E-08	3.28E-08	34	0.41	0.02
P/J	1.58E-08	1.14E-08	15	0.44	9.64E-03
DBA/1J	1.66E-08	4.09E-08	10	0.46	0.21
NOD/LtJ	1.68E-08	1.40E-08	19	0.47	0.02
A/J	2.00E-08	2.16E-08	6	0.56	0.18
C3H/HeNlcr	2.02E-08	2.48E-08	20	0.56	0.08
SWR/J	2.46E-08	3.93E-08	21	0.69	0.30
CBA/CaJ	2.94E-08	2.36E-08	12	0.82	0.51
BUB/BnJ	4.35E-08	3.39E-08	14	1.22	0.50
129/J	4.47E-08	3.21E-08	12	1.25	0.13
BALB/cAnNlcr	4.51E-08	2.72E-08	18	1.26	0.33
C57BL/10J	6.71E-08	8.58E-08	13	1.87	0.23
CAST/Ei	6.75E-08	9.61E-08	5	1.89	0.51
AKR/J	1.09E-07	1.12E-07	13	3.04	0.07
LP/J	0.00E+00	0.00E+00	3		ND

¹Metastatic index, number of metastases/lung area (μm^2)/tumor burden.

growth. Complete dissection of the tumors from a number of these animals demonstrates that the metastatic index would only be altered by 2-fold, still resulting in a highly significant reduction in the density of pulmonary metastases.

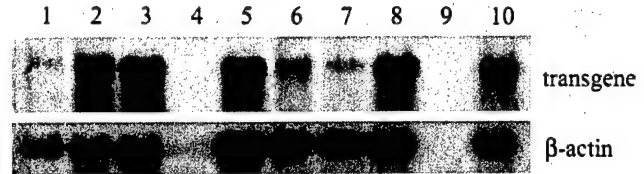


FIGURE 2 - Northern blot analysis of representative primary tumor samples. Lane 1: SEA/GnJ; lane 2: C57BL/6JNlcr; lane 3: NZB/B1NJ; lane 4: DBA/2J; lane 5: C57BR/J; lane 6: I/LnJ; lane 7: C58/J; lane 8: ST/J; lane 9: MOLF/Ei; lane 10: FVB/N. The DBA/2J and MOLF/Ei samples are degraded.

Thirteen different inbred strains were identified that had a statistically significant reduction in the metastatic index. The strains derive from many different branches of the mouse phylogenetic tree, suggesting the existence of multiple modifier genes. Additional evidence for the presence of multiple metastasis modifier genes or alleles can be inferred from the variance of the phenotype, ranging from between 2.5- to 25-fold reduction in average metastatic index compared with FVB/N. The broad range in reduction of metastatic index is most likely explained by multiple genes that were segregated into the various strains during the genesis of inbred mice. One strain, AKR/J, demonstrated a consistent approx. 3-fold increase in the metastatic index. This result is not statistically significant with the number of animals analyzed to date, but it is trending toward statistical significance. The AKR/J outcross is currently being repeated to generate enough animals to determine whether this result is significant. If significant, this inbred strain would be very useful for the discovery and analysis of dominant metastatic enhancers. Three additional strains, LP/J, RF/J and MOLF/Ei, also demonstrated a tendency toward a statistically significant reduction of metastases. However, since the number of animals scored was small, *p* values were not calculated. Outcrosses with the MOLF/Ei are being continued to evaluate the significance of the result. The LP/J and RF/J lines have been discontinued due to breeding difficulties.

Unexpectedly, in addition to dominant effects on the metastatic index, variations in the age of onset were also observed for a number of strains. The *F*₁ progeny of 2 outcrosses (I/LnJ and C58/J) had significantly shorter latency periods than the FVB/N parent. Eight additional strains had an increased latency, of approximately a week or greater. The most likely explanation for this variation would be alterations in the transgene expression, as was observed comparing other transgenic lines (Guy *et al.*, 1992). Northern blot analysis of the tumors from the *F*₁ progeny analyzed so far, however, does not demonstrate significant differences in levels of expression. Another possibility would be that the different genetic backgrounds are affecting the temporal expression of the transgene. *In situ* hybridization and immunohistochemical strategies are currently being developed in our laboratory to address this question.

The metastasis modifier genes detected in our study are likely to be generally applicable, not specific to the PyMT transgene model. In contrast to what has previously been proposed, our data suggest that expression of the PyMT transgene does not provide all the signals required for metastatic progression (Ritland *et al.*, 1997). The wide variance in metastatic phenotype between genetically identical animals and the correlation of tumor mass with metastatic density suggest that additional events must occur to permit successful tumor dissemination. These events are likely to be specific to the multiple events required to implement the metastatic pathways successfully, rather than the tumor induction mechanism and are therefore more likely to represent truly genetic interactions in human neoplastic disease. Ultimately, however, similar types of

genetic analyses will have to be performed with metastatic modifier genes as with latency modifying genes, to confirm involvement in human metastatic progression. Nonetheless, the inbred strains identified in our genetic strain survey will likely provide an extremely useful model system for the identification and characterization of the genetic modification and interactions in at least some aspects of mammary tumor neoplasia.

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Ier5, a Novel Member of the Slow-Kinetics Immediate-Early Genes

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We describe here a novel member of the slow-kinetics immediate-early gene family. *Ier5* is an intronless gene, encoding a serum- and growth factor-inducible message of 2123 nucleotides that is present in a wide variety of tissues. The predicted open reading frame encodes a 308-amino-acid, highly proline-rich protein with homology to the amino terminus of the immediate-early gene *pip92/Ier2/ETR101*. *Ier5* is predicted to be a nuclear protein and contains a PEST-like sequence, suggesting rapid protein degradation. Multiple phosphorylation sites are present. *Ier5* shows growth factor induction kinetics similar to that of *pip92/Ier2/ETR101*, but unlike *pip92/Ier2/ETR101* does not appear to require phosphokinase C activity for transcriptional activation. The sequence of the promoter region of *Ier5* was determined and examined for transcription factor binding sites thought to mediate serum and growth factor response. Multiple AP-1 sites and an Ets-1 site were observed, but the CArG and CArG-like boxes of the serum response element were absent. The predicted nuclear localization of *Ier5*, coupled with the potential for rapid regulation by phosphorylation and/or degradation, suggests that *Ier5* may play an important role in mediating the cellular response to mitogenic signals. © 1999 Academic Press

INTRODUCTION

Immediate-early genes are rapidly induced by growth factors or other stimuli whose induction is not inhibited by the presence of protein synthesis inhibitors (reviewed in Winkles, 1998). Immediate-early genes encompass members of a variety of different protein families, including members of the Fos and Jun family of transcriptional regulators, Myc, zinc-finger proteins, secreted cytokines, and cytoplasmic proteins

as well as integral membrane proteins (Winkles, 1998). Approximately 100 immediate-early genes have been described to date. Activation of the immediate-early genes is thought to be an important initial step in the regulation of cellular and genomic responses to external stimuli. Understanding the activation and coordination of this class of genes may, therefore, be important in the understanding of cellular growth control.

The immediate-early genes can be subdivided into two classes, distinguishable by their activation kinetics (Freter *et al.*, 1996). The prototype immediate-early gene, *c-fos*, is a member of the fast immediate-early genes. *c-fos* is rapidly induced after serum stimulation of quiescent cells. Transcription peaks within 30 min of activation and returns to baseline by 2 h postactivation. A number of laboratories have demonstrated that the serum response of the *c-fos* gene is mediated by three *cis*-acting regulatory elements, the serum response element (SRE), the cAMP response element, and a site for PDGF B:B homodimer binding (Freter *et al.*, 1992, 1995; Latinkic *et al.*, 1996; Shaw *et al.*, 1989; Treisman, 1990, 1992). These sites are present in a number of the fast-kinetics immediate-early genes and appear to be required for transcriptional induction.

In contrast, the slow class of immediate-early genes displays a slower induction profile following stimulation. Transcription of the slow class of genes has a greater lag and persists longer than the rapidly repressed fast-kinetics genes. These genes lack the consensus SRE binding sites, although the serum response may be mediated through degenerate or weak SRE sites (Latinkic and Lau, 1994). The lack of consensus SRE sites and the different induction kinetics suggest that the slow immediate-early genes may have activation mechanisms distinct from those of *c-fos*. Examination of the promoter sequences of the slow-kinetics immediate-early genes has failed to reveal a particular set of transcription control elements, suggesting the possibility that there may be multiple pathways that activate different members (Charles *et al.*, 1993; Chung *et al.*, 1998; Latinkic *et al.*, 1996). Among the

Sequence data from this article have been deposited with the GenBank Data Library under Accession Nos. AF079527 and AF079528.

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members of this class of immediate-early genes are the proto-oncogene *c-myc* and the monocyte chemoattractant protein 1 (MCP-1) (Freter *et al.*, 1996).

The identification and characterization of immediate-early genes have provided valuable insight into the complex pathways cells use to respond to external signals. Here we describe a novel member of the slow-kinetics class of the immediate-early gene, *Ier5*. *Ier5* encodes a proline-rich protein with multiple nuclear localization signals and amino-terminal homology to the immediate-early genes *pip92* and *ETR101*. The genomic organization of the gene and the promoter is also presented.

MATERIALS AND METHODS

Genomic subcloning and sequencing. The BAC clone 262F8 was isolated from a 129SV mouse BAC library (Birren, unpublished results) available from Research Genetics (Huntsville, AL). BAC DNA was prepared by alkaline lysis and then restriction digested to generate three *NotI* fragments. A 16-kb fragment was agarose gel-purified and subcloned into pcDNA3 and Bluescript II (KS⁻), and the ends were sequenced with the universal forward and reverse primers. This 16-kb fragment was then digested with *PstI* and/or *PvuII* to generate smaller fragments, which were then subcloned into Bluescript II KS vectors. To generate fragments suitable for sequencing from the rest of the BAC insert, BAC DNA was digested with *HindIII*, *AluI*, *HaeIII*, *RsaI*, or *BstXI* and subcloned into Bluescript KS. Additional subclones were obtained by digesting the *HindIII* clones with *PstI* and subcloning the fragments. All sequencing was performed on an ABI Model 377 automated fluorescence sequencer.

Isolation and sequencing of *Ier5* cDNA. A primer (TCTTTCTG-GACCACTGCGCA) was designed from the genomic sequence homologous to *pip92/Ier2*. The primer was used to isolate a near full-length cDNA from a mouse brain cDNA library (Stratagene) by Genetrapp (Gibco BRL) following the manufacturer's protocol. The sequence of the insert ends was determined using vector primers, and the full-length sequence was determined by primer walking. Sequencing was performed on ABI Model 377 automated fluorescence sequencer.

Growth factor response. NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum (Hyclone Laboratories), 100 μ g/ml streptomycin, 100 units/ml penicillin, and 2 mM glutamine (Life Technologies, Inc.). Upon reaching confluence, cells were switched to medium with 0.5% calf serum for 2 days and stimulated with the following agents, with and without TPA (100 ng/ml; Sigma) pretreatment overnight: serum (20%) for 30, 60, and 180 min, TPA (100 ng/ml) for 30 min, platelet-derived growth factor (50 ng/ml; Collaborative Research) for 30 min, fibroblast growth factor (200 ng/ml; Collaborative Research) for 30 min, cyclohexamide (10 μ g/ml; Sigma) for 2 h, serum (20%) in the presence of 10 μ g/ml cyclohexamide for 2 h, calcium ionophore A23187 (1 μ M; Sigma) for 2 h, cholera toxin (1.0 μ g/ml; Sigma) for 2 h, isobutylmethylxanthine (0.1 mM; Sigma) for 2 h, and a combination of isobutylmethylxanthine (0.1 mM) and cholera toxin (1.0 μ g/ml) for 30 min after stimulation of cholera toxin (1.0 μ g/ml) for 1 h and 30 min.

Northern blots. Total cellular RNA was prepared and fractionated on formaldehyde agarose gels as previously described (Sambrook *et al.*, 1989). RNAs were blotted to Hybond-N⁺ (Amersham) membranes by capillary action. Probes were prepared with the Prime-It random primer labeling kits (Stratagene) and hybridized in Church's buffer (Church and Gilbert, 1984). The blots were washed at a final stringency of $0.2\times$ SSC, 0.1% SDS at 65°C and exposed on film for 10–14 days.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16



FIG. 1. Induction of *Ier5* by serum and growth factors. NIH3T3 cells were serum starved and stimulated with serum or growth factors, and Northern blots were performed on total cellular RNA. (Top) Lanes 1; quiescent cells; 2, 30-min serum stimulation; 3, 60-min serum stimulation; 4, 180-min serum stimulation; 5, TPA; 6, TPA after 24-h pretreatment with TPA; 7, PDGF; 8, PDGF after 24-h pretreatment with TPA; 9, FGF; 10, FGF after 24-h pretreatment with TPA; 11, A23187; 12, cholera toxin; 13, cholera toxin and isobutylmethylxanthine; 14, isobutylmethylxanthine; 15, cyclohexamide; 16, cyclohexamide and TPA. (Bottom) Same filters probed with β -actin as a loading control.

RESULTS

Cloning of *Ier5*

Ier5 was identified during an unrelated positional cloning project. A 16-kb *NotI* subclone of a mouse bacterial artificial chromosome was isolated, and the ends of the insert were sequenced. BLAST (Altschul *et al.*, 1997) searches of the sequences were performed against the nucleic acid and protein databases. One end of the 16-kb subclone demonstrated significant protein homology to the amino terminus of the *pip92* (*Ier2*) gene product (Charles *et al.*, 1990) of the mouse and human transcription factor *ETR101* (Shimizu *et al.*, 1991). BLAST analysis of the expressed sequence database revealed numerous hits in a variety of tissues (embryo, uterus, placenta, mammary gland, testis, lung, kidney, and lymph node), strongly supporting the likelihood that this sequence was part of a transcription unit. An EST (Accession No. AA013648) was obtained, and the sequence was confirmed. Primers were designed from the EST sequence, and a 2-kb cDNA was isolated from a brain library by Genetrapp.

Determination of Growth Factor Response

To determine whether this transcript was a member of the set of immediate-early genes, the transcriptional response to growth factors was determined. NIH3T3 cells were serum starved and then stimulated with serum or various growth factors. Total cellular RNA was isolated, and Northern blot analysis was performed. As seen in Fig. 1, no message was detectable in quiescent cells, but a single message was observed within 30 min after exposure to serum and persisted for at least 180 min after stimulation. Transcription of the cDNA was also stimulated by TPA, FGF, and PDGF. TPA induction can be mediated through protein kinase C (PKC) substrates (Rodriguez-Pena and Rozengurt, 1985), and chronic exposure of fibroblasts to TPA has been shown to abolish PKC activity (Rodriguez-Pena and Rozengurt, 1984). To determine whether *Ier5* activation requires PKC activity, the cells were exposed to TPA for 24 h prior to growth factor

stimulation. As can be seen in Fig. 1, pretreatment with TPA did not inhibit transcriptional activation of *Ier5*, suggesting that unlike *pip92/Ier2*, PKC is not an obligate intermediate (Charles *et al.*, 1990). Cholera toxin, a cyclic AMP inducer (Cassel and Pfeuffer, 1978), or isobutylmethylxanthine, a phosphodiesterase inhibitor (Espinoza and Wharton, 1986), alone does not significantly upregulate *Ier5*; however, simultaneous treatment does result in transcriptional activation. Upregulation in response to both agents suggests that like *pip92/Ier2*, *Ier5* can be activated through a cyclic AMP-dependent pathway (Charles *et al.*, 1990). Treatment with the calcium ionophore A23187 (Nakamura, 1996) does not significantly affect expression of the transcript, suggesting that intracellular calcium levels do not play an important role in transcriptional upregulation of this gene. Transcription was upregulated by exposure to cyclohexamide, and growth factor induction was not inhibited by the presence of cycloheximide, indicating that transcriptional activation was not dependent on new protein synthesis. Since the predicted protein product bears significant homology to a portion of *pip92* and has growth factor induction profiles similar to those of other immediate-early genes, this gene, with the approval of the Mouse Genome Nomenclature Committee, has been designated *Ier5* (immediate-early gene 5).

Analysis of the cDNA and Predicted Protein Sequence

The cDNA isolated was 2074 nucleotides in length, exclusive of the poly(A) tail (see Fig. 2). The 5' end of the message including the putative open reading frame was highly GC-rich, and the 3' end is enriched for AT sequences. A single long open reading frame of 927 bases was identified, encoding a putative protein of 308 amino acids with a predicted molecular mass of 31.9 kDa. The amino-terminal 49 amino acids of the predicted protein product show significant homology to *pip92* and ETR101 (Fig. 3). The remainder of the predicted protein has no significant homology to any other molecule. Analysis by the program PSORT (Nakai and Kanehisa, 1992) predicts the presence of three potential nuclear targeting signals (PPKR, KKPR, and KPRR), suggesting that the putative protein product is a nuclear protein. The predicted protein is enriched for proline and glutamic acid, reminiscent of the PEST destruction signals in rapidly degraded proteins. Analysis of the predicted amino acid sequence with the computer program PEST-FIND (Rechsteiner and Rogers, 1996) reveals a possible PEST signal (aa 183–198, score +11.04), suggesting that like *pip92*, this protein is rapidly degraded. Several potential phosphorylation sites are also present.

Determination of *Ier5* Genomic Structure and Promoter Region

The genomic organization was determined by digesting the BAC with a variety of restriction enzymes

(*AluI*, *RsaI*, *BstXI*, *HaeIII*, *HindIII* or *PstI*), subcloning, and sequencing the fragments. The sequence of a 5447-bp region encompassing the coding region was determined. Analysis of the sequence demonstrated the presence of a putative promoter and RNA cap site 53-bp upstream of the 5' end of the cDNA. Comparison of the cDNA and genomic sequences also demonstrated that the cDNA was contiguous with the genomic DNA, with a 35-bp gap in the genomic sequence in the 3'UTR. PCR amplification with primers derived from the putative 3'UTR spanning the gap produced an identical sized product with both genomic and cDNA templates, suggesting that *Ier5* is an intronless gene.

Immediate-early genes are transcriptionally upregulated by a variety of growth factors, mediated by binding of nuclear transcription factors to sequences in the 5' untranslated region. The putative promoter sequences of *Ier5* were therefore examined with the computer program MatInspector (Quandt *et al.*, 1995) to identify any transcription factor binding sites in common with other immediate-early genes. Two possible Ets-1 sites were observed, one overlapping the translational start site. A number of potential Sp1 sites exist in the promoter region. Unlike the rapid-kinetics immediate-early genes, *Ier5* does not contain an obvious CArG box nor a CArG-like box as was observed in *pip92* and therefore does not contain an obvious SRE. Three potential AP-1 binding sites were observed, but no NF- κ B sites were found.

DISCUSSION

Ier5 is a novel member of the slow-kinetics class of immediate-early genes. This gene encodes a transcript of 2110 nucleotides in length that shares a number of nucleic acid and protein homologies to other members of the growth factor-inducible genes. As has been suggested for *pip92/Ier2*, *Ier5* is probably an intronless gene. It is unlikely that *Ier5* is a processed pseudogene due to the presence of consensus CCAAT, TATA, and GC boxes at the appropriate distance from the predicted transcriptional start site. It has been suggested that the lack of introns or the presence of relatively short introns may help promote the rapid transcriptional induction of this class of gene. Also, as has been noted for at least one other immediate-early gene, *Ier5* is in a region of high genomic GC content (60.5% for whole cDNA, 71.7% in the open reading frame) (Coleclough *et al.*, 1990). The significance of this correlation, however, is unknown.

Immediate-early gene transcripts are relatively short-lived after induction. Rapidly degraded RNAs often have AUUUA motifs that function as degradation signals (Raymond *et al.*, 1989). Examination of the *Ier5* mRNA sequence demonstrates that this degradation motif is absent, similar to what has been observed for other slow-kinetics immediate-early genes. A similar motif, GUUUG, is present (nt 1399–1403), which may substitute for the AUUUA degradation signal for

```

-2346  ggggtctttctacgaacatagataataaattagataatgtgagtatttccccacttccca
-2286  ggtagcgatgaagaactgcgtaaacataagtgggttatcacggacgaatttataaagttt
-2226  tttcaagaattttttccactgggaaagaatgcggaacacgcgtagaggcccttg
-2166  aggccctttccaaaaaagtcctttcgaagccctatagatagggtcctattcttgaaaac
-2106  cccatacctttttccgggattttatccaaacatttttggggtgggaaatagcaactaaa
-2046  tggtagctgggtttggcggttagaatggatttcaaatgcccagggggcaataagtgat
-1986  ggccaaacttatgggtcctaaggatttgggaagggaatggacagaaagataaaaaggaaa
-1926  aaaaaaacatggataaaaaaaagtcaggaaaaaccctctctataaataaataaata
-1866  aataaataaataaataaataaataaagcaggcaagtaagtaataaataaataatattt
-1806  aaaaagaagcgctgatgaaccaacaagctttgaaaggaccagggtcctgaacctgccat
      AP-1
-1746  ggctatcagacagctcactgactccacccagacggatcgctcagttggcctgtaaattta
-1686  agcgagctgtgtagacaggaacatgggatattgtctgtacactcgttcaagtgtactta
-1626  ctacgcgcatcgataaatctgtctgtgagatgcagcattacctgatacactctcacttg
-1566  taatgcactgggccccatcagagcctcactggctgtctctcttaggaacctgaagatg
-1506  aagtagggacaactctgttctttaagggtgagtggtcaggatctagatgtggtcttgga
-1446  tttcaatacccaataaataatttgcataaaaaataaaaaataaaaaagattttccaaga
-1386  gagaaaacggagaagaacctcatgctattactgaacatatgttgctgtgtacatgtta
-1326  taattattttctctctctctctatgtgggctgagaattcctagaaggaggagactctggg
-1266  cttcagttgtagcagtaatagtggccaaagttagcatcccccacacatcagatctgaag
      Ets-1
-1206  gcATCCTGgttgtgaactggctcttaataagcctacctctcactagattttctccatg
-1146  taaatggccaaacctattgtacggtcaggcaaacctttcacatagacaccggttagctg
      AP-1
-1086  tggttaactttattatttagattgactctgagagttgccatttatcttctgtgtaataaa
-1026  gaaggtgtatctgtgatttcttcattttatatttgaaacttcaggggccataaggcaattt
-966  acagagccatcaggcaccagtacaatcttgcagtggtcctctgacagggatttgcaaga
      AP-1
-906  ctcagtggtgggactacattgactgaccagaaaaatttttaggaacctccccccatact
-846  tgacaaaaaacaattttacataatcaacatgggaaaccagaacttattgtatttttagaa
-786  cttactagatttcccccaaatagatcagttacttattgtgtgtgtgtgtggggggggg
-726  tattaacaaacagacaaacaaagaaaaccattgtggagaaataggtaacatcttccatt
-666  taaaatatgtgataactgagaatctggggaacgaaatgaaagatgccagctttaggtgag
-606  aacaaacaacggtgtacactgaattctgttccctcataaggcagaagcttgggtatttcta
-546  gaacgttcccttttgtttgtagttaaagtctgagtataacgctaattgggaaagggtctaa
-486  ctccaacataatctcaaatatctgtgctagttgtgcataattgtcatcgcttccatt
-426  tctgccaaagatgaccagaaattacagctgaacacagctatgagcagctggaaattgtgta
-366  tgaaaggaaactgaattttattgtgcagtagattccagcacactaaacgaatgaaagtcc
-306  agtcagtggtgctaaggtagcatcacaaagcggaatcccttactacatacagttctgtgggc
      CAT box
-246  ctcgttctcctctatcaacgcctctgaacacaaatcccaccgcacatgatCAATgggctg
-186  cctgcaatctcccccttaagagcttgcctcggggactaacgtttgaacaggccctggg
      GC box      Sp1
-126  gccctcctctgcctctgattggtcggGGGCGGagcagcaggcacatctgcCGCCCGaga
      TATA box
-66  acggttagcggcgctttgtgattggcggttcgaggagcaTATAaagggtgcagtggtggcc

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FIG. 2. Nucleotide sequence of the *Ier5* locus. The sequence is numbered starting at the predicted cap site. The nucleotides determined from the cDNA clone are capitalized. Binding sites for regulatory elements are underlined and indicated. The predicted amino acid sequence is shown below the open reading frame. Potential phosphorylation sites are indicated by boldface font. The amino acids of the potential PEST protein degradation signal are underlined and indicated in the figure.

ETR101 (Shimizu *et al.*, 1991). In addition, there are four UUUUU sequences present in the 3' UTR that are thought to be important components of rapid RNA degradation signaling (Sokolowski *et al.*, 1997). However, mutational analysis of *c-myc*, another member of the slow-kinetics immediate-early genes, has demonstrated that RNA turnover is unaffected by deletion of the canonical degradation signals (Laird-Offringa *et al.*, 1991). It is therefore likely that *Ier5* mRNA, like other slow-kinetics immediate-early response genes, is targeted for degradation by a second AUUUA-independent pathway.

In addition to the genomic and mRNA structures,

the predicted protein of *Ier5* shares homology to another member of the immediate-early gene cohort, pip92/ETR101 (Charles *et al.*, 1990; Shimizu *et al.*, 1991). The amino terminal 49 amino acids of *Ier5* are 57% identical and 90% similar to pip92/ETR101. This region, which is predicted to form two α helices separated by a short intervening sequence, may form an important structural domain (Fig. 3). The similarity between the amino termini of *Ier5* and pip92/ETR101 suggests that these proteins may interact with similar sites, either by protein-protein or protein-nucleic acid interaction. *Ier5* contains multiple nuclear localization signals (ProArgLysArg, LysLysProArg, LysProArgArg)

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      0          20          40
-6  cgcagcgtcagtttagagtccggctcagaacggttctgttGTCTGAAGGGAAGCGGCGG

      60          80          100
    AGGGGGACTACGCGTCACCAGGTCTTTTCTCCACCGAGCTTCAGACGCCGAGGGTACAA

      120          140          160
    CCACAGAGGAAGACCTGATCCCGCTACAGCTCCTCTCGCAGCTTCAGAAGGCAAGCAGG

      180          200          220
    CGTTACACCTATCGTCTGGAAGCGAAGGCAGGATGGAGTTCAAGCTGGAGGCTCACCGCA
      MetGluPheLysLeuGluAlaHisArgI

      240          260          280
    TCGTCAGCATCTCCCTGGGCAAGATCTACAACCTCGCGGGTCCAGCGCGCGGCATCAAGC
    leValSerIleSerLeuGlyLysIleTyrAsnSerArgValGlnArgGlyGlyIleLysL

      300          320          340
    TGCACAAGAACCTCTTGGTGTCTGGTGTCTGCGCAGCGCCCGCAAGTCTACCTGAGTG
    euHisLysAsnLeuLeuValSerLeuValLeuArgSerAlaArgGlnValTyrLeuSerA

      360          380          400
    ACCCCTGCCCTGGCCTCTACCTGGCTGGCCCTGCGGGGACCCCGCGGTGCCGCCGCTC
    spProCysProGlyLeuTyrLeuAlaGlyProAlaGlyThrProAlaValProProProG

      420          440          460
    AGCAGCCTGGAGAGCCTGTAGCCGGGCCACCCTCCGGCTGGGGAGAGCCCGCCCGCGG
    lnGlnProGlyGluProValAlaGlyProProSerGlyTrpGlyGluProProProProV

      480          500          520
    TCGCCCGTGCCGCTGGCCGGAGCCCGAGCCTCAGCCGAGCCTCAGCCTCAGCGTCCCT
    alAlaArgAlaAlaTrpProGluProGluProGlnProGlnProGlnProGlnArgProS

      540          560          580
    CCGTGTGCCACACGCCCCGAGCGGGAAGCTCAGAACCGGTGGCCGCGGTGGCCGGATCGG
    erValCysHisThrProGlyAlaGlySerSerGluProValAlaAlaValAlaGlySerG

      600          620          640
    GGGAAAGCTCTCCGGGCGGAGAGGAGGACTCGCGCGCAGCTGCTTGGGGCCGCTGGAGC
    lyGluAlaLeuArgGlyGlyGluGluAspSerAlaAlaAlaAlaTrpGlyArgValGluA

      660          680          700
    GTCCGCGCGCGGCGTCTTCAGGAGGTGGCTCGGACGCGTGCCCCGAGGGGCCCCGGGCAG
    rgProArgAlaAlaSerSerGlyGlyGlySerAspAlaCysProGluGlyProArgAlaV

      720          740          760
    TGCGCCGCGCGTGGCGCTGTCCCCGGCCGTGGAGGAGCGGTCTCCGAGGACGGGTCCC
    alArgArgProCysGlyCysProProAlaValGluGluArgSerSerGluAspGlySerP
      PEST Sequence

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FIG. 2—Continued

and, like ETR101, is probably a nuclear protein. Multiple potential phosphorylation sites are present, similar to ETR101, suggesting possible posttranslational activation or regulation. *Ier5* also contains a PEST-like sequence and, like *pip92/Ier2*, is probably rapidly degraded. *Ier5* may function as a transcriptional regulator, possibly by binding to DNA or by mediating nuclear protein-protein interactions. The potential to degrade and/or phosphorylate *Ier5* may therefore be important for the rapid control of signals transmitted through the *Ier5* pathway. However, since *Ier5* shares homology with no protein other than *pip92/Ier2*/

ETR101, and only over the first 49 amino acids, the function of these molecules remains obscure.

Serum response of the *c-fos* and other fast-kinetics immediate-early genes is mediated by the SRE, composed of an Ets-like binding site (CAGGAT) in either orientation upstream of a CArG box [CC(A/T)₆GG] (Latinkic and Lau, 1994). The slow-kinetics immediate-early genes lack canonical SREs, but often have Ets-like and CArG-like sites that are thought to be responsible for the serum response (Latinkic and Lau, 1994). The promoter region of *Ier5* was therefore examined to determine whether similar sites were


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      780              800              820
CGCGCCACCCGCGGCTGTCCAGGAAGCGTGGCGCAGCGGGAGTGGGTGGCGGCCGCG
roAlaProProAlaProCysProArgLysArgGlyAlaAlaGlyValGlyGlyGlyArgA

      840              860              880
CGAGCTGCTCGGCACCCGGCTCTACCCCTCTCAAGAAGCCGCGCCGGAAGTCCGGAGGAGC
laSerCysSerAlaProGlySerThrProLeuLysLysProArgArgAsnSerGluGluG

      900              920              940
AGCCAGTCACCGGCGAGGACGACACCGACGAGGAGATGGAGACCGGGAACGTGGCAAACC
lnProValThrGlyGluAspAspThrAspGluGluMetGluThrGlyAsnValAlaAsnL

      960              980              1000
TCATCAGCATCTTCGGGTCTAGCTTCTCGGGACTCCTACGAAAAGCCCCGAGCGGCC
euIleSerIlePheGlySerSerPheSerGlyLeuLeuArgLysSerProAlaGlyGlyA

      1020             1040             1060
GAGAGGAAGAGGAGGCGGAGGAGGGCGGCCCGGAAGCCGCGAGCCGGGGCAGATCTGCT
rgGluGluGluGluAlaGluGluGlyGlyProGluAlaAlaGluProGlyGlnIleCysC

      1080             1100             1120
GCGACAAGCCGGTCTGAGAGACATGAGCCCCTGGAGCACAGCCATCGTGGCCTTCTGAG
ysAspLysProValLeuArgAspMetSerProTrpSerThrAlaIleValAlaPheEnd

1134 CCCCCGGGACCCGAGTGGCTGGAGCTTGAGCAGCGGGAGTCCCCGAAGTGGCGCCGGGGC
1194 CTCTCCCGGCCGCGAGGACGAGCCGAGACCGTAGGCGCTGGGCGCGTAGGGGAGACTGGT
1254 TGCTGCCGGGCAGCACATCGCCCCAGAAGGGGCAGCCCGGCTGGGCGCTGGCTCTCGAGT
1314 CTCATCCTGAGACCTAGAGTTGGAGGCTTATTGGAAGAGGACGATCGATCGTTAACTTTT
1374 GTATCTGTGTGTGTCGCTGTGTAAGTTGTCTTGTGTGAGACTGTTCTGTGATTCTGGA
1434 ATGCACCACCCCTTCCCCGGGGCTTGAGACTTTGGGGGTGCAGACAGGGACTTTCTCTG
1494 CCAGCAGCACAGAAAAGGAAGCTGCAGAAAGGCTCGAGGAAGGGGAGTTCCCGTTCGAT
1554 CTTGGGGGAGAGAGGGACTTTACACACACCTCTCTCATGAAAGCTACGACCGCACCCGG
1614 GCGCGGAGTGGCGTTTTCTCACAGGATTTCTCAGACTAGAGGGAGTTTCGTCTGGAGTA
1674 GAGACTTGTTATTTTCTCCCTCCCCATCCTTTCTCTGGCACAGAAGAAGCGCTTCTA
1734 CCCTGTGATTACTCTGGGAAAGCAGAACATTAAGGGACCTTCAATTCCCCACAGGGGAAA
1794 AAAAGCTTGCTGAACCTTCACAGAAGAGTTCAAGCTTGGAATACGGAGTTTATAGAGAAA
1854 AGATATATTTTTTAAAGCTCTAGCTCAGAACTTACTACACAGTGCTTTTAAAGTGTT
1914 TAATGAAACAAAGTTTACAGACGAGCTCTAAGTGAAGGACCTTATGGCTTTGTAGATAA
      PDGF site

1974 GGTGACTCCAGTCTTTGTTGTATAAAGGTTGGGGGAGCTGATAAGGTTTGTACAGTAT
2034 TTTCTCCTTCTCTGTATTGATTTTGTATAAAATGTAACCTTACGTGTCTAACACGTAT
2094 TAAATATTTTGAAGCAactccactgctgtttgtatgtatgtaatcacttgctggtttt
2154 ccaacaaggtggcgaggagggtaccaggctgggaatggaaggtgtggtcctcaatat
2214 ccccatcaccaggcgaggagcagtggttgactgaataactaccaacaagacacg
2274 ttttaagggtttggttttgggtttttgttttcacgaacatccccatccatggatactgga
2334 tcaatgactgtcagtttgacacttgccactacaattttctcttcattcacgacaacacag
2394 tttggtttcctatactttgatctgacgggaatctctaattattacacaatattaaagcca
2454 aattttgcagcctttccaatatcaaataatttccacaaaactttgtttccacaataagtc
2514 tggtcacgtatagatcgatatattaatctctacactccaattgggggtgctgggggttaca

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FIG. 2—Continued

present that would account for the transcriptional activation observed upon serum or growth factor stimulation. Two Ets-1 sites were observed, one overlapping the translational start site and one in the 5' controlling region of *Ier5*. Neither site appears to be accompanied by a CARG-like sequence. It has been proposed that the serum response of at least two other slow-kinetics immediate-early genes is mediated by high-affinity Ets sites in conjunction with a low-affinity CARG box (Latinkic *et al.*, 1996). The sequence that functions as a CARG box would therefore not necessarily be easily recognizable by simple sequence analysis. Examination

of the sequence, however, does not predict any of the CARG-like sequences observed in the *pip92/Ier2* promoter since no (A/T)₆ motifs are present. It is possible that there is a degenerate sequence present that significantly deviates from the CARG or CARG-like sequences that accounts for the serum response. Alternatively, like *MCP-1* and *c-myc*, *Ier5* may not contain *fos*-like SREs (Freter *et al.*, 1992). Functional studies of the promoter activity will be required, however, to determine the validity of these hypotheses.

The presence of Ets sites has been shown to be important for the induction of immediate-early genes by

FIG. 2—Continued

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Microsatellite DNA variants among the FVB/NJ, C58/J, and I/LnJ mouse strains

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Introduction

The availability of high-resolution mouse genetics and the large number of well-characterized inbred strains make the mouse a powerful system for studying multigenic or quantitative trait human disease phenotypes (Frankel 1995). The advent of transgenic mice has provided a model system to study the genetic basis of many inheritable traits and processes. The inbred mouse strain, FVB/NJ, is widely used for the creation of transgenic animals owing to the large male pronucleus, which permits easy visualization and DNA microinjection (Taketo et al. 1991).

The utility of FVB/NJ for genetic mapping studies is limited by the lack of information regarding DNA variant alleles between this and other inbred laboratory mouse strains. Neuhaus et al. recently reported variations between FVB/NJ and C3HeB/FeJLe and C57BL/6J mouse strains (Neuhaus et al. 1997). We performed an analysis of the FVB/NJ, C58/J, and I/LnJ mouse strains DNA with 419 microsatellite primers along the 20 mouse chromosomes and report the variant alleles in this paper.

Materials and methods

FVB/NJ, C58/J, and I/LnJ females were obtained from The Jackson Laboratory (Bar Harbor, Me.). Genomic DNA was obtained from livers and purified. The DNA was then amplified by PCR with microsatellite primers developed at the Whitehead Institute/MIT Center for Genome Research and obtained from Research Genetics (MAPPAIR™ Primers from Research Genetics, Huntsville, Ala.). An aliquot of 20 ng of genomic DNA was amplified in 20-μl PCR reaction containing 2 μl 10× PCR buffer, 0.4 μl 10 mM dNTPs, 0.6 μl 25 mM MgCl₂, 1 μl of each primer, 0.1 μl 5U/μl Taq Polymerase, and 14.9 μl sterile water. The PCR reaction was carried out in a thermal cycler (MJ Research PTC-200, Watertown, Mass.) as follows: 1 cycle 95°C for 2 min; 35 cycles 94°C for 45 s, 57°C for 45 s, 72°C for 60 s; a final elongation cycle 72°C for 7 min. PCR products were then run on a 4% agarose gel and analyzed for polymorphisms. Utilizing this technique allows for the identification of polymorphisms greater than 6–10 base pairs.

Results

Variant alleles between FVB/NJ and C58/J and I/LnJ mouse strains were analyzed with a set of 419 microsatellite primers along the 20 mouse chromosomes. The average spacing between primer sets along each chromosome was 3.5 cM. Of the 419 primers used, approximately 10% failed to amplify. We identified 73 (19%) markers that were variant between FVB/NJ and C58/J, and 80 (21%) markers variant between FVB/NJ and I/LnJ. Since our resolution was only 6–10 base pairs, there are likely to be more polymorphic loci that could not be identified. These variations are expressed in Table 1 as a function of the chromosome studied. A

Table 1. Results.

Locus	Location	FVB	C58	I/Ln
D1Mit33	82	*	>	>
D1Mit36	91.8	*	*	>
D1Mit46	43.7	*	*	>
D1Mit231	8.7	*	*	<
D1Mit303	32.8	*	*	>
D1Mit356	94	*	<	<
D1Mit362	110.4	*	*	<
D2Mit75	48.1	*	>	*
D2Mit92	42.6	*	*	<
D2Mit194	66.7	*	*	<
D2Mit274	52.5	*	*	>
D2Mit277	56.8	*	>	>
D2Mit304	59	*	*	>
D3Mit29	33.9	*	*	>
D3Mit45	57.9	*	*	<
D3Mit46	12	*	*	<
D3Mit224	16.4	*	*	>
D3Mit230	28.4	*	*	>
D4Mit178	30.6	*	<	*
D4Mit204	61.2	*	<	*
D4Mit214	21.9	*	>	>
D4Mit234	71	*	*	>
D4Mit251	66.7	*	*	<
D5Mit30	62.3	*	>	>
D5Mit81	18.6	*	*	<
D5Mit223	77.6	*	*	>
D5Mit240	43.7	*	<	*
D5Mit259	32.8	*	<	*
D5Mit429	77.6	*	<	*
D6Mit8	25.1	*	<	<
D6Mit14	63.4	*	<	<
D6Mit15	66.7	*	>>	>
D6Mit123	17.5	*	>	*
D6Mit243	19.7	*	>	*
D6Mit259	56.8	*	<	*
D6Mit261	29.5	*	*	<
D7Mit44	50.3	*	*	<
D7Mit76	3.3	*	*	<
D7Mit220	38.3	*	>	*
D7Mit232	26.2	*	*	<
D7Mit246	12	*	*	>
D7Mit253	41.5	*	*	>
D7Mit259	67.8	*	<	*
D8Mit141	4.4	*	*	<
D8Mit191	21.9	*	*	<
D8Mit215	61.2	*	>	>
D8Mit249	38.3	*	>>	>
D9Mit82	67.8	*	<	<
D9Mit182	53.6	*	*	<
D9Mit191	20.8	*	>	*
Dd9Mit207	31.7	*	<	*
D9Mit212	60.1	*	<	*
D10Mit83	4.4	*	*	<
D10Mit95	50.3	*	*	>
D10Mit117	44.8	*	>	>
D10Mit186	36.1	*	*	>
D11Mit4	36.1	*	>	>
D11Mit71	0	*	<<	<

Table 1. Continued.

Locus	Location	FVB	C58	I/Ln
D11Mit99	60.1	*	>	*
D11Mit132	62.3	*	<	*
D11Mit149	2.2	*	*	<
D11Mit214	77.6	*	*	<
D11Mit229	10.9	*	*	>
D11Mit231	14.2	*	*	<
D12Mit10	5.5	*	>	<
D12Mit134	57.9	*	*	>
D12Mit156	28.4	*	<	<
D12Mit172	17.5	*	>	*
D12Mit182	2.2	*	*	<
D12Mit201	23	*	*	<
D13Mit16	5.27	*	*	>
D13Mit78	59	*	>	*
D13Mit171	50.3	*	<	<
D13Mit191	29.5	*	>	*
D13Mit202	32.8	*	*	<
D13Mit207	2.2	*	*	<
D13Mit213	40.4	*	<	*
D14Mit141	24	*	>	*
D14Mit165	62.3	*	>	*
D14Mit170	69.9	*	<	>
D14Mit194	52.5	*	>	>
D14Mit195	52.5	*	<	<
D14Mit196	57.9	*	>	>
D14Mit203	37.2	*	<	*
D15Mit159	49.2	*	>	>
D15Mit174	0	*	>	*
D15Mit175	5.5	*	>	*
D15Mit179	7.7	*	*	>
D15Mit184	24	*	*	>
D15Mit187	32.8	*	*	<
D15Mit193	57.9	*	<	<
D16Mit4	25.1	*	<	*
D16Mit32	0	*	<	<
D16Mit49	39.3	*	>	*
D16Mit51	50.3	*	<	*
D16Mit131	6.6	*	<	*
D16Mit132		*	<	*
D16Mit154	3.3	*	>	*
D17Mit16	7.7	*	>	*
D17Mit81	5.5	*	<	<

Table 1. Continued.

Locus	Location	FVB	C58	I/Ln
D17Mit93	39.3	*	<	<
D17Mit123	50.3	*	<	*
D17Mit139	25.1	*	>	>
D17Mit177	19.7	*	<	<
D18Mit19	0	*	>	*
D18Mit21	3.3	*	*	>
D18Mit25	39.3	*	>	*
D18Mit42	45.3	*	<	>
D18Mit142	31.7	*	*	>
D18Mit149	15.3	*	>	*
D19Mit46	24	*	>	*
D19Mit68	3.3	*	>	*
D19Mit71	55.7	*	<	<
D19Mit79	8.7	*	<	<
D19Mit88	24	*	>	>
DXMit140	20.8	*	>	>
DXMit141	20.8	*	>	*

The FVB DNA product was used as the standard, and results are expressed in relation to the size of this project. ">" represents a product that is larger than FVB, while "<" is one which is smaller. A double sign, ">>" or "<<" is used to differentiate the larger or smaller product between the C58 and I/Ln strain.

full list of the microsatellite primers used can be found at www.resgen.com.

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An Epistatic Interaction Controls the Latency of a Transgene-Induced Mammary Tumor

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Previous studies from our laboratory have demonstrated that the latency, tumor growth and metastatic progression of polyoma middle T-induced mammary tumor in an FVB/NJ inbred mouse background could be significantly altered by the introduction of different genetic backgrounds. In this study we extend these findings by mapping a number of interacting quantitative trait loci responsible the changes in phenotype.

Introduction of the I/LnJ inbred genetic background into the FVB/NJ-PyMT animal significantly accelerates the appearance of the primary tumor (35 vs. 57 days postnatal, $p < 10^{-7}$). A backcross mapping panel was established and loci responsible for the tumor acceleration were detected on chromosomes 15 and 9. Examination of the genotype/phenotype correlation revealed that the FVB/NJ but not the I/LnJ allele of the chromosome 15 locus was associated with tumor acceleration, and was conditional on the presence I/LnJ allele on chromosome 9. These loci, designated *Apmt1* and *Apmt2* map to syntenic regions associated with LOH in human breast cancer. These results suggest that in addition to loss of function, allelic variants of genes in these regions may contribute to age of onset in human breast cancer.

Introduction

The inherited component of breast cancer was originally observed more than 100 years ago [1], and has been confirmed by the identification of two major susceptibility genes, *BRCA1* [2] and *BRCA2* [3]. Women with germline mutations in these genes have a greatly enhanced risk of breast cancer. The likelihood of women carrying mutations in *BRCA1* developing breast cancer ranges from approximately 50-80% by the age of 70 [4, 5] and carriers are also at increased risk for ovarian cancer [6]. Mutations in *BRCA2* appear to confer equal risk of breast cancer [7] but do not confer a significantly elevated risk of ovarian cancer [8]. Approximately 70% of breast cancer families can be accounted for by the high susceptibility genes [9] and between 10-15% of breast cancer in the general population [10, 11].

Although the discovery of these high susceptibility genes is important for understanding the genetic basis of breast cancer, many important questions remain to be answered. The clinical expression of *BRCA1* has been shown to vary both between families and within families [4, 12, 13]. Some women may develop breast cancer early in life, whereas family members bearing the same mutation may remain unaffected until their seventies [14]. The variable penetrance and age-at-onset observed between individuals bearing the same mutations suggests that there must be additional factors that influence the development of the disease. For example, over-expression of the estrogen receptor has been suggested to be a risk factor [15]. An allelic variant of the *CYP19* gene (aromatase p450) has been found significantly more frequently in breast cancer patients than in control patients and has led to the suggestion that *CYP19* is a susceptibility gene with low penetrance [16]. Rare alleles of the *HRAS1* gene have also been shown to modify the risk of breast cancer [17]. The genes responsible for hereditary non-polyposis colorectal cancer (*HPNCC*), the candidate tumor suppresser gene of Cowden disease [18], and the ataxia-telangiectasia gene [19] have also been associated with breast cancer. Allelic variation in these putative susceptibility genes may, therefore, contribute to the genetic complexity of this disease.

Identifying and characterizing genes that modify breast cancer risk would provide important new insights into the etiology of breast cancer [20]. At present, however, little is known about breast cancer modifier genes and how they interact with the major susceptibility genes. Due to the genetic heterogeneity of the human population and interactions with uncontrolled environmental influences, identification of modifiers in human populations can be a difficult task. Because of these difficulties, the mouse has often been used as a model for many human disorders that have both simple and complex genetic components. The mouse has been used successfully for the genetic analysis of many different phenotypes including epilepsy, diabetes, obesity, pigmentation, lupus, alcohol or drug preference, as well as cancer (reviewed in [21]). The availability of large numbers of inbred strains, high-resolution mapping reagents, and the ability to dissect complex qualitative and quantitative traits make the mouse a valuable tool for analysis of the complex genetic basis of mammary carcinogenesis.

Previously we reported the identification of a number of mouse inbred strains in which the initiation and progression of a transgene induced mammary tumor was significantly different from the typical pattern [22]. FVB/N-TgN(MMTVPyVT)634Mul mice bear a Polyoma Middle T antigen gene that is expressed under the control of a mouse mammary tumor virus enhancer and promoter [23]. Female animals inheriting this transgene develop palpable, synchronous multifocal mammary tumors in all of the mammary glands at approximately 60 days of age [23]. F₁ progeny of a cross between the FVB/N-TgN(MMTVPyVT)634Mul and the inbred strain I/LnJ, however, have an average latency of only 36 days of age. These data strongly suggest the presence of mammary tumor susceptibility alleles in the I/LnJ strain. We therefore initiated both genetic and biochemical studies to assess the basis for the tumor acceleration in the I/LnJ F₁ animals. We

demonstrate that the change in tumor latency cannot be explained by alterations in expression of the transgene and thus the alteration in tumor latency is most likely due to an increased sensitivity of the I/LnJ mammary gland to polyoma middle T-induced tumorigenicity. Quantitative trait locus (QTL) analysis of an I/LnJ backcross identified at least two interacting genetic regions associated with the increased susceptibility to mammary tumors. None of the genes previously associated with breast cancer susceptibility map to these chromosomal intervals. The I/LnJ alleles may therefore represent novel breast cancer susceptibility genes.

Materials and Methods

Animals. FVB/N-TgN(MMTVPyVT)634Mul mice were obtained from W. Muller, McMaster University, Hamilton, Ontario, Canada. I/LnJ mice were purchased from The Jackson Laboratory. The backcross (N₂) animals were generated by breeding FVB/N-TgN(MMTVPyVT)634Mul males to I/LnJ females and crossing the transgene-positive males F₁ back to FVB/NJ females. Inheritance of the polyoma transgene was determined by PCR amplification of weanling tail biopsy DNA with the following primers: 5'-AAC GGC GGA GCG AGG AAC TG-3'; 5'-ATC GGG CTC AGC AAC ACA AG-3'. Diagnosis of mammary tumors was performed by palpation. Animals were checked for tumors every other day. After the initial identification of the primary tumor, animals were further aged to confirm the diagnosis.

Northern Blot Analysis. Total RNA was isolated from the tumors using an RNAeasy kit (Qiagen). RNAs were fractionated on formaldehyde TBE gels, transferred to Hybond N+ membranes by capillary action and probed with a randomly primed PyMT probe.

Antisera. Monoclonal rabbit anti-Polyoma T Antigen antibodies were obtained from Oncogene Research Products. Polyclonal rat anti-polyoma T antigen antibodies were kindly provided by T. Benjamin (Harvard University, Cambridge, MA).

Western Blot Analysis. Tumors were homogenized in lysis buffer (1 x PBS, 1% Triton x100, 0.5% deoxycholate, 0.1% SDS, 0.004% NaF, 100 µg/ml PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 2 mM NaOrthovanadate, pH 7.4) and centrifuged at 100,000 g to pellet insoluble material. The protein concentration of the supernatant was determined by the BCA method (Biorad). Western blots were performed basically as described [24].

Immunohistochemistry. Tissues were fixed in neutral buffered formalin, paraffin embedded and sectioned. Immunohistochemical stains were performed with a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA), following the manufacturer's protocol.

Genotyping. Tail biopsy DNA was used as a template for PCR reactions. Microsatellite primers were purchased from Research Genetics (Huntsville, AL). PCR reactions were performed basically as described [25]. Reactions were performed in a PTC200 Thermocycler (MJ Research, Watertown, MA) and analyzed on 4% agarose TAE gels. The following loci were used: D1Mit1, D1Mit33, D1Mit46, D1Mit105, D2Mit1, D2Mit113, D2Mit277, D3Mit29, D3Mit147, D3Mit224, D4Mit9, D4Mit17, D4Mit18, D4Mit200, D4Mit214, D4Mit256, D4Mit308, D4Mit348, D5Mit81, D5Mit201, D5Mit223, D5Mit247, D6Mit14, D6Mit15, D6Mit123, D6Mit138, D7Mit44, D7Mit76, D7Mit109, D7Mit232, D7Mit246, D8Mit191, D8Mit215, D9Mit82, D9Mit129, D9Mit182, D9Mit207, D9Mit355, D10Mit16, D10Mit95, D10Mit186,

D11Mit4, D11Mit214, D11Mit231, D12Mit10, D12Mit134, D12Mit201, D13Mit16, D13Mit171, D13Mit202, D14Mit120, D14Mit170, D15Mit105, D15Mit179, D15Mit184, D15Mit193, D16Mit32, D16Mit139, D17Mit81, D17Mit93, D17Mit139, D17Mit177, D18Mit60, D18Mit142, D19Mit71, D19Mit79, D19Mit88, DXMit140.

Data Analysis. The dataset was mapped using Map Manager QT [26]. Appropriate statistical thresholds ($p = 0.5, 0.05$, and 0.01) for mapping QTLs [27] were estimated by permuting the correctly ordered dataset 10,000 times using the Doerge and Churchill [28] algorithm implemented by Map Manager. Each permutation was mapped at 1 centimorgan intervals across the entire genome.

Statistical Analysis. The epistatic interaction of the two loci was assessed under the assumption that each locus has a predominantly additive effect on response (latency). An interaction between loci would then mean that the magnitude of the additive effect at one locus depends on the level of the other locus. This hypothesis was formally tested via analysis of variance. The dependent variable was tumor latency and the additive main effect of each individual locus was modeled through an indicator variable for locus genotype. The main effect of each locus (the additive effect of one locus on latency averaged over the levels of the other locus) as well as the interaction between the two loci was then estimated and tested. Physically, the interaction term represents the difference in the additive effect of one locus at the two levels of the other locus. Additional statistical analysis was performed with Quick Statistica Package (Statsoft, Tulsa, OK).

Results

Analysis of Transgene Expression in Mammary Tumors. To determine whether the change of the tumor latency in the I/LnJ F₁ animals was due to alterations in the expression level of the PyMT antigen in the tumors, Northern blots were performed. Total RNA was isolated from FVB/N-TgN(MMTVPyVT)634Mul and [I/LnJ x FVB/N-TgN(MMTVPyVT)634Mul]F₁ tumors and sequentially probed with a polyoma middle T probe and b-actin as a loading control. No difference in the transcription level of the polyoma middle T mRNA was observed between FVB/NJ and the [I/LnJ x FVB/NJ]F₁ tumors (see Fig. 1).

In order to rule out the possibility that the change in tumor latency was due to a difference in post-transcriptional control of the transgene, Western blots were performed. Total protein was isolated from tumors and probed with anti-polyoma T antibodies. As can be observed in Fig. 2, no difference in the levels of the polyoma middle T antigen was observed between the FVB/N or [I/LnJ x FVB/NJ]F₁ tumors. In addition, no evidence for differential post-translation modification was observed.

Determination of Transgene Temporal Expression. The acceleration of tumor latency might also have been explained by different temporal expression patterns of the transgenes in the different genetic backgrounds. To address this possibility, immunohistochemical analyses were performed. Previous studies from other laboratories demonstrated the presence of pathological abnormalities present in the mammary glands of 21-day old FVB/N animals, indicative of transgene expression at that age. Therefore, mammary glands from 5 and 10 day old transgene-positive female [I/LnJ x FVB/NJ]F₁ females were harvested, sectioned and *in situ* immunohistochemical stains with the anti-polyoma middle T antibodies performed. No expression of the middle-T antigen was detected in the 5-day old mammary gland (data not shown). Middle-T protein was detected in the 10 day old gland (see Fig. 3). Some, but not all of the epithelial

ducts stained with the antibody. In addition, some of the ducts showed sectorial expression (see Fig. 3). These data suggest that the transgene was being translationally activated in the mammary epithelium of the 10 day old [I/LnJ x FVB/NJ] F_1 animals. Similar expression patterns of the middle-T antigen was observed in 10 day old transgene-positive FVB/NJ animals, demonstrating that the acceleration of the [I/LnJ x FVB/NJ] F_1 tumors could not be simply explained by earlier expression of the transgene.

QTL Mapping. Taken together, these data strongly suggested that the acceleration of the mammary tumorigenesis observed in the [I/LnJ x FVB/NJ] F_1 animals was due to the presence of a dominant mammary tumor susceptibility allele(s) in the I/LnJ background. A backcross was generated to identify the genomic region or regions that harbored the susceptibility gene(s) (Fig. 4). Transgene-positive FVB/NJ males were bred to I/LnJ females and the transgene-positive F_1 males crossed back to FVB/NJ females. The reciprocal backcross was not performed due to the possibility of recessive alleles in the I/LnJ background that might have interfered with the analysis. 126 female backcross (N_2) animals were generated, the tumor latency determined, and a genome scan performed with 69 loci. The haplotype and latency data were analyzed with the computer program Map Manager QT to identify loci associated with the acceleration of the tumor latency. One interval that exceeded the suggested statistically significant threshold (LOD = 3.3; genome-wide $p = 0.01$) was identified near the D15Mit184 microsatellite locus (Fig. 5). This locus was responsible for approximately 7% of the variance observed. We have designated this locus Accelerator of Polyoma-Induced Mammary Tumors (*Apm1*). A second locus on chromosome 7 approached but did not exceed the recommended statistically significant threshold. A third suggestive interval was identified on chromosome 9 near the locus D9Mit182.

Analysis of Epistatic Interaction. Unexpectedly, examination of the genotype by phenotype correlation of *Apm1* in the extreme 25% of the latency distribution demonstrated an over-representation of the slow latency FVB/NJ allele in the fast arising tumors (23/31) and an exclusion of the FVB/NJ allele from the long latency tumors (10/31; $\chi^2 = 10.9$, 1 d.f., $p = 0.0009$). This suggested the possibility that the change in tumor latency was generated in large part by an epistatic interaction of an unknown I/LnJ allele with the FVB/NJ allele of *Apm1*. Genotype data were reexamined to discover heterozygous loci that were strongly associated with tumor acceleration in FVB/NJ animals that were likely to be *Apm1* homozygotes. There was a strong correlation between tumor latency and the interval on chromosome 9 (see above). Animals that were heterozygous for *D9Mit182* and homozygous for *D15Mit184* had the greatest acceleration of the tumor compared to FVB/NJ parents (38.67 days versus 56.63 days, $p < 10^{-7}$, see Table 1). All four genotype classes were significantly different than the FVB/NJ parent, indicating the presence of at least one additional modifier gene. However, no other combinations that we examined demonstrated such a significant association. Therefore we have designated the chromosome 9 locus *Apm2*.

The effect of the *Apm1* and *Apm2* interaction was determined by comparing the effect of the four genotype classes to each other. As can be seen in Table 2, animals homozygous for both loci were not significantly different from animals homozygous for chromosome 9 and heterozygous for chromosome 15 (row A). Conversely, there was a significant difference between the double heterozygotes and the Chr 9 heterozygous / Chr 15 homozygous animals (row B). These data suggest that *Apm1* on chromosome 15 acts in an additive manner with each

FVB/NJ allele accelerating tumor latency approximately 10 days, but is conditional on the presence of an I/LnJ allele at *Apm2*.

This putative interaction was further explored by analyzing the role of the chromosome 9 locus in the chromosome 15 homozygous subset of animals. If the Chr. 15 locus was dependent upon the presence of an I/LnJ allele on Chr. 9, the Chr. 9 locus would be expected to be highly significant in that population. The effect of the Chr. 9 locus was therefore examined in the 63 Chr. 15 homozygous animals. A Chi-squared analysis demonstrated that the association of the I/LnJ allele on Chr. 9 with tumor latency was highly significant ($p = 0.00009$) and accounted for 20% of the variance in this population.

The putative epistatic interaction was formally analyzed using analysis of variance. If the effect of each locus on tumor latency was primarily additive and there was no epistatic interaction, then the difference in the latencies associated with the distinct genotypes at one locus would be the same for each genotype of the other locus. That is, the difference in the latencies of the Chr. 9 FF/Chr. 15 FF and Chr. 9 FI/Chr. 15 FF is expected to be the same as that between the Chr. 9 FF/Chr. 15 FI and Chr. 9 FI/Chr. 15 FI animals. Assuming that latency had approximately the same variance for each of the genotypes (supported by the fact that the sample standard deviations only ranged from 7.5 to 12.9), analysis of variance can then be used to assess an interaction between the two loci by testing the equality of the two differences. Since this results in the difference among Chr. 15 FF animals (the estimated difference is $49.4 - 38.7 = 10.7$ days) being declared significantly greater than the difference among Chr. 15 FI animals (estimated as $50.8 - 48.6 = 2.2$ days; $p = 0.03$), we conclude that the loci appear to interact with respect to their effect on tumor latency. Together, the interacting loci account for approximately 17% of the variance observed.

Discussion

The intended goal of this project was to identify genes that had a dominant effect on the ability of the transgene-induced mammary tumor to form. In previous studies, we had performed a strain survey to identify inbred strains, including I/LnJ, whose genomes would significantly alter the initiation or progression of the tumor in F_1 hybrid animals. We therefore had anticipated that the gene or genes mapped in the current study would be of I/LnJ origin acting in a simple dominant manner. The identification that the FVB/NJ allele *Apm1* on Chr. 15 was the only locus that exceeded the suggested statistically significant threshold for an additive interaction. This locus however required the presence of at least one I/LnJ allele elsewhere in the genome that was interacting with *Apm1*. Since we had performed a backcross it was possible to identify *Apm2* on Chr. 9. An intercross would have required the examination of nine different genotype classes instead of the four produced in the backcross. It is unclear whether we would have been able to easily identify *Apm2* with the number of animals used.

There are two possible models that would explain the genetic interaction observed in this study. The first model would be that the Chr. 9 locus was dependent on a recessive allele at the Chr. 15 locus, and only exerted its effect when Chr. 15 was homozygous for the FVB/NJ allele. The other model would be that the Chr. 15 FVB/NJ allele acts additively on latency, but only in the presence of an I/LnJ allele on Chr. 9. We favor the latter explanation for the following three reasons. First, the acceleration of latency was observed originally in an F_1 population. Therefore the major modifier loci can not be operating as recessives. Secondly, the *Apm1/Apm2* compound heterozygous animals, which are most analogous to the F_1 population, are highly significantly different than the FVB/NJ parents ($p = 0.002$, see table 1) suggesting that these loci play a significant role in the acceleration of the disease. However, since these animals do not have

as severe a phenotype as the F₁ population there are almost certainly other modifiers present, probably including the Chr. 7 locus. Finally, if the Chr. 15 locus was recessive, then there would have to be other major modifier loci to explain the tumor acceleration observed. Only the three loci described, on chromosomes 7, 9, and 15 were observed in our analysis, even at the low stringency genome-wide *p* threshold of 0.05, arguing against the presence of additional major modifier loci. Thus, although there are likely to be other possible explanations, we feel the most likely model would be that the second model proposed, i.e. the FVB/NJ allele of *Apm1* on Chr. 15 interacts additively with the I/LnJ allele of *Apm2* on Chr. 9.

The acceleration of the tumor latency in the I/LnJ animals was of particular interest because of the known sensitivity of I/LnJ mammary glands to hormones [29]. Explanted I/LnJ mammary glands exposed to exogenous hormones in organ cultures were shown to develop more rapidly than other inbred strains. Endogenous hormone levels are thought to play an important role in the development of breast cancer [30, 31]. In addition, use of exogenous hormones [32, 33], oral contraceptives [34] have also been implicated in the etiology of breast cancer. We therefore determined whether any of the hormones or hormone receptors important for mammary development mapped to the *Apm1* or *Apm2* candidate regions. The estrogen receptor α and β chains are on chromosomes 10 and 12 respectively (MGD, <http://informatics.jax.org>). *Cyp19* and *Cyp11a*, the enzymes that catalyze the biosynthesis of estrogen and progesterone are on Chr. 9 but located proximal of the *Apm2* candidate region. The prolactin gene is on Chr. 13. The prolactin receptor maps to the proximal end of Chr. 15, but is not included in the *Apm1* candidate region. The progesterone receptor gene maps to human chromosome 11q22, which is syntenic with proximal mouse Chr. 9. This region however is not contained in the *Apm2* candidate region and therefore excludes the progesterone receptor as a candidate for *Apm2*. Combined, these data suggest that the acceleration of tumor latency in the I/LnJ animals is not due to a direct affect of these hormones or receptors, although an indirect effect has not been ruled out.

We have examined a number of other loci commonly associated or suspected to be associated with breast cancer as potential candidates for *Apm1* and *Apm2*. *Brca1*, *Brca2*, *Trp53*, and *ErbB2*, genes associated with hereditary breast cancer or genetic abnormalities in sporadic cancers, map to Chr. 11. *Brca2* is located on Chr. 5. *Hras1* is on Chr. 7. These loci are therefore not responsible for the increased susceptibility of the I/LnJ mice to polyoma middle T-induced mammary tumors. *Myc*, which is often amplified in breast cancer [35] is located within the *Apm1* candidate region and therefore might be considered a candidate locus.

Since we have excluded most of the known or suspected breast cancer susceptibility loci, *Apm1* and *Apm2* may therefore represent novel breast cancer susceptibility genes. However, polyoma virus, the proximal cause of tumors in this model, to the best of our knowledge, is not associated with breast cancer. It is possible that the loci detected in this study are specific to the polyoma middle T pathway and have no role in human cancer. While it is not possible at this time to rule out this possibility, it is encouraging to note that the *Apm1* and *Apm2* candidate regions are syntenic with human chromosomal regions commonly deleted in breast cancer (8p and 3p, respectively) [36, 37]. Ultimately the identification of these genes will be required before an unambiguous answer about their role in human cancer can be determined. Towards this end we are currently pursuing several different strategies, including the generation of congenic mouse strains to further delineate the *Apm1* and *Apm2* candidate regions, to identify these modifiers of breast cancer induction.

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Table 1: Comparison of FVB/NJ, F₁ and N₂ Latencies

<u>Locus/Genotype</u>	<u>FVB/NJ</u>	<u>I/LnJ F₁</u>	<u>N₂ Genotypes</u>			
D9Mit182 (<i>Apmt2</i>)	-	-	FF	FI	FF	FI
D15Mit184 (<i>Apmt1</i>)	-	-	FF	FF	FI	FI
Average Latency	56.6	37.3	49.4	38.7	50.8	48.6
N	108	35	28	34	30	34
P value	1	<10 ⁻⁷	0.01	<10 ⁻⁷	0.04	0.002

P values were determined by comparing the latency data of each genotype class against the FVB/NJ parent with the Mann Whitney U test and corrected for multiple tests.

Table 2: Comparisons of Ampt1/2 Genotype Classes

		A.	B.	
		D9Mit182/D15Mit184	D9Mit182/D15Mit184	P value
A.	Genotypes	FF/FF	FF/FI	
	Average Latency	49.4	50.8	0.43
B.	Genotypes	FI/FF	FI/FI	
	Average Latency	38.7	48.6	0.0003
	P value	0.0003	0.49	

P values were determined by comparing the latency data of each genotype class with the Mann Whitney U test and corrected for multiple tests.

Figure Legends:

Figure 1: Northern blot of FVB/NJ and [I/LnJ x FVB/NJ] F_1 tumors. No difference in mRNA expression was observed between the two genotypes.

Figure 2: Comparison of Tumor Latency with transgene expression in N_2 animals. Total protein extracts prepared from tumors probed with antibody against the polyoma middle T antigen. The latencies of the animals, in days after birth are as follows: lane 1: 74 days; lane 2: 74 days; lane 3: 80 days; lanes 4-6: 29 days. The upper band in lane 3 is an artifact and is not reproducible

Figure 3: Example of Polyoma Middle T immunostain of 10 day old mammary glands. Adjacent serial sections of paraffin embedded mammary glands stained with or without an anti-PyMT monoclonal antibody. **A:** I/LnJ F_1 mammary gland, no antibody control; **B:** I/LnJ F_1 mammary gland, with PyMT antibody; **C:** FVB/NJ mammary gland, no antibody control; **D:** FVB/NJ, PyMT antibody. The arrows indicate PyMT staining of the glandular epithelia.

Figure 4: Backcross Design. Transgene-positive FVB/NJ males were mated to I/LnJ females. The transgene-positive male F_1 animals were then backcrossed to transgene-negative FVB/NJ females to generate the N_2 population.

Figure 5: Results of the genome wide QTL scan for an additive model. The strategy is described in the text. The heavy horizontal line depicts the chromosomes with the centromeres to the left. The loci scored are listed below the chromosome. The dashed lines represent the statistically significant thresholds. The lower line represents the suggestive and the upper the significant threshold. **A:** Chromosome 7; **B:** Chromosome 9; **C:** Chromosome 15.